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Nucleic acids

THE NUCLEIC ACIDS

Chemistry and Biology

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Volume I

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Preface

There are many mansions in the house that the Natural Sciences have been building in the course of 400 years; but it has remained one edifice. Although its architectural style may not be of one piece, combining, as it does, the labors of so many generations, the very consistency of the structure makes difficult the coherent description of one wing without a distortion of proportions. An arbitrary line of demarcation must, however, be drawn in a book of the kind we are presenting here.

The progress in our knowledge of nucleic acids has been unexpectedly rapid in the past few years, and there is no reason to assume that it will not continue. Until recently, the state of nucleic acid chemistry could be compared to that in which protein chemistry found itself at the beginning of this century. The principal constituents were known, at least in the two nucleic acids that served as the prototypes; but a real insight into the order and mode of their arrangement was lacking. All this has changed; the nucleic acids are no longer regarded as mere pegs for the proteins. The large number of publications, the multiplication of partial efforts, of fragmentary reviews and symposia, make imperative an attempt at a detailed inventory of our knowledge.

This treatise tries to collect all the information at present available into a single comprehensive work. It is divided into two main parts, covering the chemical and the more biological or biochemical aspects of the subject, respectively. The chemistry of the hydrolysis products of nucleic acids is covered in Chapters 2, 3, and 4, and their separation and estimation in Chapters 5 to 9. The two main types of polynucleotides are discussed in Chapters 10 and 11. The nature of the chemical bonds in nucleic acids is considered in Chapter 12, their physical properties in Chapter 13, and their optical properties in Chapter 14. The principal absorption spectra provided in Chapter 14 are also reproduced, on a metric scale, on two folded sheets that will be found in a pocket on the inside back cover of Volume I.

The next chapter (15), which deals with the enzymes attacking nucleic acids, forms the connecting link with the second main division of the book which begins with a survey of the nucleic acid content of tissues, in Chapter 16, and of cytochemical methods, in Chapter 17. The cell nucleus is discussed in Chapters 18, 19, and 20, and the cytoplasm in Chapter 21. The next four chapters cover the biosynthesis of nucleic acids and their components. Nucleic acid metabolism is discussed in Chapter 26, and the book ends with two chapters on the biological role of the two main types of nucleic acid (Chapters 27 and 28).

A collaborative undertaking, the only one possible at the present time, entails well-known dangers. The Editors dare not hope that they have evaded them, but in designing the book they have, as far as lay in their power, made a determined effort to avoid producing a mere collection of essays or review articles. Instead they have attempted to provide a continuous narrative in which the tale, although taken up by one storyteller after another, nevertheless has a continuous and connecting thread running through it from beginning to end. There may be gaps in the narrative, parts of the story may be told more than once, but a sincere attempt has been made to help the reader by linking the chapters with abundant cross references. And it must not be forgotten that, even in a book of this size, the story is far from complete, for additions to our knowledge of the nucleic acids are made available with almost every issue of a host of scientific journals.

The Editors owe a deep debt of gratitude to the many contributors who have made the treatise possible and to the publishers for their unfailing help and advice. They also wish to thank their secretaries, Mrs. Emmy Bloch and Miss Mary Gilmour, for their patient and willing help.

If this book helps create an early need for a supplement to its present content, it will have fulfilled one of its purposes.

ERWIN CHARGAFF
J. N. DAVIDSON

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CHAPTER 1

Introduction

J. N. DAVIDSON AND ERWIN CHARGAFF

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> Die Würdigung Miescher's und seiner Arbeiten wird mit der Zeit nicht abnehmen, sondern wachsen, und die von ihm gefundenen Thatsachen und gedachten Gedanken sind Keime, denen noch eine fruchtbringende Zukunft bevorsteht.
>
> W. His. May, 1897

I. The Early History

The discovery of the nucleic acids was the result of the work of Friedrich Miescher (1844–1895), the founder of our knowledge of the chemistry of the cell nucleus. As a pupil of Hoppe-Seyler in Tübingen in 1868–69, Miescher became interested in the problem of isolating nuclear components, choosing as his source of material the pus cells which he obtained from the surgical bandages discarded in the nearby surgical clinics. By digesting the cells with pepsin–hydrochloric acid and then shaking with ether, he was able to isolate the nuclei as a separate layer which settled to the bottom of the vessel and could be filtered off. From the nuclear material he was able to prepare a hitherto unknown compound which he called "nuclein." This substance was acidic in nature, readily soluble in dilute alkali but insoluble in dilute acid, and contained a high proportion of phosphorus. This last property alone was sufficient to attract attention to the compound for at that time the only known organic compound of phosphorus in the tissues was lecithin.

Miescher submitted an account of his results to Hoppe-Seyler, who found them so surprising that he hesitated to publish them in his journal until he had himself repeated the work, but in 1871 Miescher's original account together with Hoppe-Seyler's confirmation and supplementary papers by two of his pupils appeared in *Hoppe-Seyler's Medicinisch-chemische Untersuchungen*.[1]

---

[1] F. Miescher, *Hoppe Seyler's Med. chem. Unters.* **1871,** 441; P. Plosz, *ibid.* **1871,** 461; N. Lubavin, *ibid* **1871,** 463; F. Hoppe-Seyler, *ibid* **1871,** 486; F. Miescher, *ibid.* **1871,** 502.

By this time Miescher had returned to Basel in his native Switzerland where he found a more congenial and convenient source of nuclear material in the sperm heads of the Rhine salmon. From these he isolated a high-molecular-weight nuclein and a basic material less complex than known proteins which he called "protamine" and which could be extracted from the defatted sperm with dilute acid leaving the "nuclein" in the residue. This nuclein had a phosphorus content of 9.59 % and gave analytical figures corresponding to what is now known as nucleic acid. Indeed Miescher's many preparations of nucleins from a variety of sources, carried out at low temperatures under the most exacting conditions, would be regarded by present day standards as highly satisfactory. In subsequent work he showed that the nucleins of the salmon sperm were synthesized at the expense of the musculature of the fish, which do not eat during the period of gonadal development in fresh water.

After Miescher's death his friends, in particular His, organized the publication of a collected edition of his works including many of his letters.[2] His work was continued by his successors. For example, Altmann,[3] who first used the term *nucleic acid*, developed methods for the preparation of protein-free nucleic acid from yeast as well as from animal tissues, while Kossel and Neumann[4] described a method for its preparation from thymus glands. These methods of preparation were subsequently improved and developed by Neumann.[5]

The discovery of the purine bases in nucleic acids was made by Piccard,[6] who at Miescher's suggestion extracted salmon sperm with boiling hydrochloric acid and isolated guanine and hypoxanthine. But the most outstanding work on the purine bases was carried out by Kossel,[7] who for several years from 1879 onwards was actively engaged in this field and was responsible for the isolation of xanthine and adenine.

Miescher had isolated from the products of the hydrolysis of nucleic acid a base which we now know to have been thymine, but this pyrimidine was not properly identified until the work of Kossel and Neumann in 1894.[4] Cytosine was isolated and identified in 1902–03 by Kossel and Steudel[8] and by Levene[9] from thymus nucleic acid, while Ascoli[10] in 1900 isolated uracil from yeast nucleic acid.

---

[2] "Die histochemischen und physiologischen Arbeiten von Friedrich Miescher," 2 vols. F. C. W. Vogel, Leipzig, 1897.

[3] R. Altmann, *Arch. Anat. u. Physiol., Physiol. Abt.* **1889,** 524.

[4] A. Kossel and A. Neumann, *Ber.* **27,** 2215 (1894).

[5] A. Neuman, *Arch. Anat. u. Physiol., Suppl.* **1899,** 552.

[6] J. Piccard, *Ber.* **7,** 1714 (1874).

[7] A. Kossel, *Z. physiol. Chem.* **3,** 284 (1879); **5,** 152 (1881); **6,** 422 (1882); **7,** 7 (1882–3); **8,** 404 (1883–4); **10,** 248 (1886); **12,** 241 (1888).

[8] A. Kossel and H. Steudel, *Z. physiol. Chem.* **37,** 177 (1902–3).

II. The Two Types of Nucleic Acid

The work of these early pioneers in nucleic acid chemistry, Miescher, Kossel, Neuman, Steudel, O. Hammarsten, and others, is fully described in the books by Jones,[11] Feulgen,[12] and Levene and Bass,[13] and no more need be said about it here. By 1930, however, a definite picture had emerged of two definite types of nucleic acid. One of them, the nucleic acid from yeast, on hydrolysis yielded adenine, guanine, cytosine, uracil, phosphoric acid, and a sugar recognized by O. Hammarsten as a pentose and identified by Levene and Jacobs[14] as ribose. The other, the nucleic acid from thymus, yielded adenine, guanine, cytosine, thymine, phosphoric acid, and a sugar at first thought to be a hexose but later shown by Levene[15] to be a deoxypentose and identified as deoxyribose.[16] These two nucleic acids, therefore, came to be called *ribonucleic acid* and *deoxyribonucleic acid*, respectively, and, since most nucleic acids of animal origin appeared to resemble that from thymus, while the triticonucleic acid isolated from wheat embryo by Osborne and Harris[17] was similar to that from yeast, the assumption was made that pentose nucleic acids were characteristic of plants and deoxypentose nucleic acids of animal tissues.[11, 13] The terms phytonucleic acid and zoonucleic acid were suggested for these two groups, respectively.[13]

This classification, however, was never free from objection since it had been known from the end of the last century that pentose derivatives were present in animal tissues. In 1894, for example, O. Hammarsten[18] prepared from pancreas tissue a "β-nucleoprotein" from which Bang[19] obtained a

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[9] P. A. Levene, *Z. physiol. Chem.* **37,** 402 (1902–3).

[10] A. Ascoli, *Z. physiol. Chem.* **31,** 161 (1900–1901).

[11] W. Jones, "Nucleic Acids—Their Chemical Properties and Physiological Conduct," 2nd ed. Longmans Green & Co., London, 1920.

[12] R. Feulgen, "Chemie und Physiologie der Nukleinstoffe," Borntraeger, Berlin, 1923.

[13] P. A. Levene and L. W. Bass, "Nucleic Acids." Chemical Catalog Company, New York, 1931.

[14] P. A. Levene and W. A. Jacobs, *Ber.* **42,** 2102, 2469, 2474, 2703 (1909).

[15] P. A. Levene and E. S. London, *J. Biol. Chem.* **81,** 711 (1929); **83,** 793 (1929).

[16] P. A. Levene, L. A. Mikeska, and T. Mori, *J. Biol. Chem.* **85,** 785 (1930).

[17] T. B. Osborne and I. F. Harris, *Z. physiol. Chem.* **36,** 85 (1902).

[18] O. Hammarsten, *Z. physiol. Chem.* **19,** 19 (1894).

[19] I. Bang, *Z. physiol. Chem.* **26,** 133 (1898–99); **31,** 411 (1900–1901).

"guanylic acid" which could be precipitated by acetic acid. This material was subsequently shown by the work of Feulgen,[20] of E. Hammarsten,[21] of Hammarsten and Jorpes,[22] and Jorpes[23-25] to be a pentose polynucleotide. From such pancreas material also, Jones and Perkins[26] isolated, in crystalline form, the pentose nucleotides of adenine, guanine, and cytosine and reported the presence in spleen and liver of a substance resembling the guanylic acid of the pancreas. It was at this time (1924) that the idea began to develop of a more widespread occurrence of pentose nucleic acids in animal tissues than had previously been supposed.

It had been known for a long time that, in addition to the coenzyme nucleotides, pentose nucleotides presumably derived from polynucleotides were present in animal tissues such as chick embryo,[27] mammary gland,[28, 29] haddock[30] and sea urchin[31] eggs, and even spleen and liver.[26, 32] All this evidence led to the suggestion made by Jones and Perkins[26] in 1924 and supported by Jorpes[24] in 1928 that "the distinction between animal and plant nucleic acid will in future not be so definitely drawn." Further support for this view was provided by evidence for the presence of deoxyribonucleic acids in plant tissues.[33-36]

As the result of the development of Brachet's ribonuclease test [Chapter 17], the presence of pentose nucleic acids was demonstrated histochemically in amphibia,[37-40] in the anterior pituitary of the growing rat and guinea pig,[41] and in toad's eggs.[42] At the same time the pioneer work of the Caspersson

[20] A. Feulgen, *Z. physiol. Chem.* **108,** 147 (1919–20).

[21] E. Hammarsten, *Z. physiol. Chem.* **109,** 141 (1920).

[22] E. Hammarsten and E. Jorpes. *Z. physiol. Chem.* **118,** 224 (1922).

[23] E. Jorpes, *Biochem. Z.* **151,** 227 (1924).

[24] E. Jorpes, *Acta Med. Scand.* **68,** 253, 503 (1928).

[25] E. Jorpes, *Biochem. J.* **28,** 2102 (1934).

[26] W. Jones and M. E. Perkins, *J. Biol. Chem.* **62,** 290 (1924–25).

[27] H. O. Calvery, *J. Biol. Chem.* **77,** 489 (1928).

[28] R. Odenius, *Jahresber. Fortschr. Thierchem.* **30,** 39 (1900).

[29] J. A. Mandel and P. A. Levene, *Z. physiol. Chem.* **46,** 155 (1905).

[30] P. A. Levene and J. A. Mandel, *Z. physiol. Chem.* **49,** 262 (1906).

[31] K. C. Blanchard, *J. Biol. Chem.* **108,** 251 (1935).

[32] P. Thomas and C. Berariu, *Compt. rend. soc. biol.* **91,** 1470 (1924).

[33] R. Feulgen and H. Rossenbeck, *Z. physiol. Chem.* **135,** 203 (1924).

[34] A. Kiesel and A. N. Belozerski, *Z. physiol. Chem.* **229,** 160 (1934).

[35] A. N. Belozerski, *Biokhimyia (U. S. S. R.)* **1,** 253 (1936); *Compt. rend. acad. sci. U. R. S. S.* **25,** 751 (1939).

[36] M. Behrens, *Z. physiol. Chem.* **253,** 185 (1938).

[37] J. Brachet, *Arch. biol. (Liége)* **44,** 519 (1933).

[38] J. Brachet, *Arch. biol. (Liége)* **48,** 529 (1937).

[39] J. Brachet, *Arch. biol. (Liége)* **51,** 151, 167 (1940).

[40] J. Brachet, *Compt. rend. soc. biol.* **133,** 88, 90 (1940).

[41] L. Desclin, *Compt. rend. soc. biol.* **133,** 457 (1940).

[42] T. S. Painter and A. N. Taylor, *Proc. Natl. Acad. Sci. U. S.* **28,** 311 (1942).

school[43-45] with the quantitative ultraviolet spectrophotometric technique [Chapter 17] led to the detection of the presence of high concentrations of pentose nucleotides or pentose polynucleotides in the cytoplasm of rapidly proliferating cells such as sea urchin eggs, the spinach root-tip periblem cell, the imaginal disks of larvae of *Drosophila melanogaster*,[46, 47] embryonic tissues,[46, 48] tumor tissues,[49] and the cells of actively secreting glands.[50] From all these results Caspersson[45] concluded that a high concentration of pentose nucleic acid was characteristic of cells in which rapid protein synthesis was taking place, either for growth or for secretion.

These conclusions, based on histochemical and spectrophotometric methods, were confirmed for embryonic tissues in 1943 by Davidson and Waymouth,[51, 52] who showed by chemical methods that pentose nucleic acid was abundant in a large number of tissues in the sheep embryo and more abundant than deoxypentose nucleic acid in many. The pentose nucleic acid, however, was not peculiar to embryonic tissues for it was also found in a corresponding series of adult tissues. Although the total nucleic acid was in most cases higher in the embryo than in the adult, the amount of pentose nucleic acid relative to deoxypentose nucleic acid varied from tissue to tissue and was of the same order in embryonic as in the corresponding adult tissue. The pentose nucleic acid of sheep liver was isolated in 1944 and shown to be present in liver tissue in the surprisingly large proportion of 3 or 4 times as much pentose nucleic acid as deoxypentose nucleic acid.[53] Its sugar was identified conclusively as D-ribose.[54] Since then, pentose nucleic acids have been isolated from many animal tissues. Their properties and the methods of isolation are discussed in Chapter 11. The deoxypentose nucleic acids are discussed in Chapter 10.

Although the nucleic acids were originally thought to be essentially nuclear constituents, the occurrence of the pentose type in the cytoplasm was suspected as long ago as 1905.[55] In deciding the location of the nucleic acids

[43] T. Caspersson, *Skand. Arch. Physiol.* **74,** suppl. 8 (1936).

[44] T. Caspersson, *J. Roy. Microscop. Soc.* **60,** 8 (1940).

[45] T. Caspersson, *Naturwissenschaften* **29,** 33 (1941).

[46] T. Caspersson and J. Schultz, *Nature* **143,** 602 (1939).

[47] T. Caspersson and J. Schultz, *Proc. Natl. Acad. Sci. U. S.* **26,** 507 (1940).

[48] T. Caspersson and B. Thorell, *Chromosoma* **2,** 132 (1941).

[49] T. Caspersson, C. Nyström, and L. Santesson, *Naturwissenschaften* **29,** 29 (1941); *Acta Radiol., Suppl.* **46,** (1942).

[50] T. Caspersson, H. Landström-Hyden, and L. Aquillonius, *Chromosoma* **2,** 111 (1941).

[51] J. N. Davidson and C. Waymouth, *Nature* **152,** 47 (1943).

[52] J. N. Davidson and C. Waymouth, *Biochem. J.* **38,** 39 (1944).

[53] J. N. Davidson and C. Waymouth, *Biochem. J.* **38,** 379 (1944).

[54] J. N. Davidson and C. Waymouth, *Biochem. J.* **38,** 375 (1944).

[55] S. P. Beebe and B. Shaffer, *Am. J. Physiol.* **14,** 231 (1905).

in the cell, histochemical tests have been of great value [Chapter 17]. For example, the Feulgen nucleal reaction,[33] which is specific for deoxypentose nucleic acid, has been used to demonstrate that this type of nucleic acid is confined to the cell nucleus. Similarly the Brachet[37-39] histochemical test with ribonuclease [Chapter 17] has demonstrated the presence of pentose nucleic acid in the cell cytoplasm, in obviously abundant amounts in such tissues as liver.[56] In Caspersson's ultraviolet technique no distinction is made between the two types of nucleic acid, but material which absorbs ultraviolet light strongly and which is Feulgen-negative is considered to be pentose polynucleotide.

The general conclusions from histochemical tests have been confirmed by the procedure of cell disruption with separation of the morphological components of the cells, as described in Chapters 18 and 21. The cytoplasmic components contain pentose nucleic acid while the nuclei contain deoxypentose nucleic acid and small amounts of pentose nucleic acid [Chapter 18].

We may therefore conclude that both types of nucleic acid are present in all types of cell, both plant and animal, and that the main biological distinction between pentose nucleic acid and deoxypentose nucleic acid is that the former is mainly cytoplasmic while the latter is exclusively, or almost exclusively, nuclear. The designations "chromonucleic" and "plasmonucleic acids" have been proposed for deoxypentose and pentose nucleic acids, respectively,[57, 58] but these names are not widely used.

The study of nucleic acids entered a new stage when the application of paper chromatography to the separation of nucleic acid constituents (purines, pyrimidines, nucleosides, nucleotides) made possible precise analytical investigations with very small amounts of material[59-62] [Chapters 7, 10, and 11]. The introduction of ion-exchange chromatography [Chapter 6] represented another important step. All these techniques were instrumental in the development of current conceptions of nucleic acid structure [Chapters 10–12] and helpful in the rapid advance in synthetic procedures for the preparation of nucleic acid constituents [Chapter 4].

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[56] J. N. Davidson and C. Waymouth, *Proc. Roy. Soc. Edinburgh* **B62,** 96 (1944).

[57] A. W. Pollister and A. E. Mirsky, *Nature* **152,** 692 (1943).

[58] A. W. Pollister and A. E. Mirsky, *Nature* **153,** 711 (1944).

[59] E. Vischer and E. Chargaff, *J. Biol. Chem.* **168,** 781 (1947).

[60] E. Vischer and E. Chargaff, *Federation Proc.* **7,** 197 (1948).

[61] R. D. Hotchkiss, *J. Biol. Chem.* **175,** 315 (1948).

[62] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176,** 703, 715 (1948).

[63] E. Chargaff, *Experientia* **6,** 201 (1950); *J. Cellular Comp. Physiol.* **38,** suppl. 1, 41 (1951); *Federation Proc.* **10,** 654 (1951).

[64] E. Chargaff and E. Vischer, *Ann. Rev. Biochem.* **17,** 201 (1948).

[65] J. N. Davidson, *Ann. Rev. Biochem.* **18,** 155 (1949).

[66] G. Schmidt, *Ann. Rev. Biochem.* **19,** 149 (1950).

[67] J. Baddiley, *Ann. Rev. Biochem.* **20,** 149 (1951).

[68] D. O. Jordan, *Ann. Rev. Biochem.* **21,** 207 (1952).

[69] G. B. Brown, *Ann. Rev. Biochem.* **22,** 141 (1953).

[70] J. N. Davidson, "The Biochemistry of the Nucleic Acids," 2nd ed. Methuen, London, 1953.

It has now become abundantly clear that the names pentose nucleic acid and deoxypentose nucleic acid are generic terms indicating groups of compounds of similar composition. Chargaff and his colleagues[63] were the first to show that there are many nucleic acids differing in composition as regards molar proportions of bases according to the biological source of the material from which they are derived. There is even evidence of heterogeneity within the cell, for the pentose nucleic acids of the cytoplasm appear to differ slightly from those of the nucleus in the same tissue [Chapter 11] while the deoxypentose nucleic acids of the nuclei of a single cell type have been separated into fractions of different molar composition [Chapter 10].

The pentose sugar has been identified as ribose in the pentose nucleic acid of yeast[14] and of liver[53] and has been shown to be chromatographically identical with ribose in the nucleic acids from a large number of other sources [Chapter 11]. Consequently, such nucleic acids are frequently referred to as ribonucleic acids (RNA) instead of pentose nucleic acids (PNA). Since there is no evidence of the presence of any other pentose sugar, both terms are legitimate, but for the sake of uniformity the contraction PNA will be used in this book.

The sugar in thymus deoxypentose nucleic acid has been conclusively proved to be D-2-deoxyribose[16] and the sugars in the corresponding nuclear nucleic acids from a large number of other tissues have been shown to be chromatographically identical with it [Chapter 10]. Consequently, these nucleic acids are frequently referred to as deoxyribonucleic acids; but, whether they be called deoxypentose nucleic acids or deoxyribonucleic acids, the contraction DNA is conveniently and frequently used for both names.

III. Previous Literature

No major treatises on the chemistry of nucleic acids have appeared since those of Jones,[11] Feulgen,[12] and Levene,[13] nor has the biochemistry of nucleic

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[71] R. Vendrely, *Bull. soc. Chim. biol.* **32,** 427 (1950).

[72] F. Schlenk, *Advances in Enzymol.* **9,** 455 (1949).

[73] Nucleic acid, *Symposia Soc. Exptl. Biol.* **1** (1947).

[74] Nucleic acids and nucleoproteins, *Cold Spring Harbor Symposia Quant. Biol.* **12** (1947).

[75] Symposium on the biochemistry of nucleic acids, *J. Cellular Comp. Physiol.* **38,** suppl. 1 (1951).

[76] The chemistry and physiology of the nucleus, *Exptl. Cell Research Suppl.* **2** (1952).

[77] P. Boulanger and J. Montreuil, *Bull. soc. chim. France,* **1952,** 844.

[78] A. E. Mirsky, *Sci. American* **188,** 47 (1953).

acids and of their constituents been considered in detail. A large number of monographs, reviews, and symposia has, however, been published, a selection of which is listed here.[64-82]

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[79] The chemistry and metabolism of nucleic acids, *Phosphorus Metabolism* **2,** 301–439 (1952).

[80] Symposium on nucleoproteins, *Can. J. Med. Sci.* **31,** 222–302 (1953).

[81] J. Brachet, "Le rôle des acides nucléiques dans la vie de la cellule et de l'embryon." Desoer, Liége, and Masson, Paris, 1952.

[82] F. Egami, "Nucleic Acids and Nucleoproteins, Physics, Chemistry, Biology and Medicine." 2 vols. Kyoritsu Pub., Tokyo, 1951.

CHAPTER 2

Chemistry of Ribose and Deoxyribose

W. G. OVEREND AND M. STACEY

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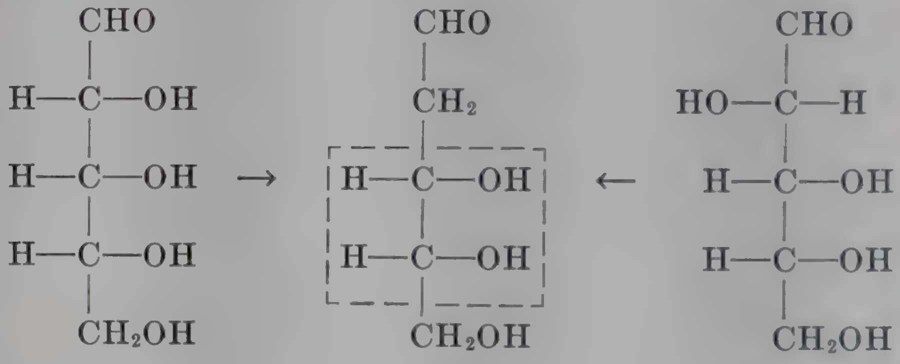
I. Introduction

In recent years knowledge of the chemistry of the sugar components of nucleic acids has been considerably broadened. Methods have been developed for the synthesis of important derivatives of these sugars, and the properties and reactions of the compounds thereby obtained have been investigated. In particular the general chemistry of 2-deoxyribose has been intensively studied. Many experiments with D-ribose have had as objective the preparation in good yield of intermediates suitable for use in projected syntheses of naturally occurring ribo-nucleosides and -nucleotides. Several excellent reviews have been published in recent years describing the chemistry of ribose,[1] of sugar phosphates including the phosphoric acid esters of ribose and deoxyribose[2] and of ribo(and deoxyribo)-nucleosides and -nucleotides.[3] In addition a recent description of the chemistry of 2-deoxysugars[4] includes much of interest concerning 2-deoxyribose.

At the outset of this account attention must be directed to the nomenclature used for the class of compounds known as deoxysugars. A brief perusal of the literature shows that it is extremely confusing. (See Overend and Stacey[4] for a full discussion of this problem.) For example, at various times the sugar component of thymus nucleic acid has been referred to by several different names. It is usual to name a deoxy-pentose or -hexose after the parent sugar from which it is prepared. The sugar moiety of thymus nucleic acid can be prepared from D-ribose (I) by the glycal reaction—which is used for the synthesis of 2-deoxysugars—and therefore may be named 2-deoxy-D-ribose (II). However, due to the loss of asymmetry at carbon atom 2 during this reaction, II could be prepared from D-arabinose (III) by the same series of reactions, and so could equally well be referred to as 2-deoxy-D-arabinose. This latter name has been preferred by some chemists. Recently it has become possible to prepare II from D-erythrose[5, 6] without using either D-ribose or D-arabinose as an intermediate, so that at first sight this system of nomenclature might



I II III

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[1] R. W. Jeanloz and H. G. Fletcher, Jr., *Advances in Carbohydrate Chem.* **6,** 135 (1951).

[2] L. F. Leloir, *Fortschr. Chem. org. Naturstoffe* **8,** 47 (1951).

[3] G. W. Kenner, *Fortschr. Chem. org. Naturstoffe* **8,** 96 (1951).

[4] W. G. Overend and M. Stacey, *Advances in Carbohydrate Chem.* **8,** 45 (1953).

[5] J. C. Sowden, *J. Am. Chem. Soc.* **71,** 1897 (1949); **72,** 808 (1950).

[6] W. G. Overend, M. Stacey, and L. F. Wiggins, *J. Chem. Soc.* **1949,** 1358.

ppear to be outmoded. Besides minor variations, such as the use of both deoxyribose'' and "desoxyribose,'' a trivial name—"thyminose''—has een adopted by some authors. Although this trivial name is found rarely owadays in chemical literature, it persists to some extent in contemporary ublications relating to the biological sciences.

Attempts have been made to systematize the nomenclature of deoxy- ugars. Bergmann and his co-workers[7] introduced the ending "desose'' o denote a deoxysugar and referred to II as ribodesose. Probably the best uggestion for systematizing the names of deoxysugars was made by Sow- en,[8] who proposed that the stereochemistry of the hydroxyl groups in eoxy-pentoses and -hexoses should be denoted by a prefix. On this system I is named D-*erythro*-2-deoxypentose. The dotted outline denotes the ortion of the sugar molecule to which the prefix relates.

Athough from the chemical viewpoint this latter system of nomenclaure is probably the most satisfactory, it is not proposed to use it in this eview and instead II will be referred to as 2-deoxy-D-ribose. In this way ontinuity and familiarity will be maintained with the nomenclature dopted in the majority of papers relating to aspects of the chemistry of I. In a survey summarizing the chemistry of ribose and deoxyribose, it s considered to be important that changes in nomenclature from that enerally adopted by previous authors should be minimized.

Although this account will be concerned primarily with a description f the chemistry of D-ribose and 2-deoxy-D-ribose, with particular emphasis n those aspects of the subject which relate directly to nucleic acids, reference will be made to other pentose derivatives whenever they serve to llustrate by analogy or comparison the main theme of this chapter.

II. Occurrence of D-Ribose and 2-Deoxy-D-Ribose

The only sugars found so far as components of nucleic acids are D-ribose I) and 2-deoxy-D-ribose (II).

Although Kossel[9] recognized in 1891 that acidic hydrolysis of what is ow called ribonucleic acid liberates a carbohydrate derivative and three ears later Hammarsten[10] demonstrated that this sugar component was a entose, some fifteen years elapsed before Levene and Jacobs[11, 12] succeeded n isolating the sugar in crystalline form and identifying it as D-ribose, at hat time an unknown sugar. Its identification was based on the facts that t differed in its physical constants from the crystalline pentoses (L-arabinose, D-xylose and D-lyxose) known at that time: that its benzylphenylhydrazone differed from that of arabinose;[13] but the melting point and numerical value (though not the sign) of the specific rotation of its osazone and *p*-bromophenylosazone were the same as for these derivatives of L-arabinose: that on oxidation it gave D-ribonic acid, enantiomorphous with that previously prepared synthetically by Fischer and Piloty,[14] and that on further oxidation it yielded an inactive trihydroxyglutaric acid. Final complete verification was achieved when Alberda van Ekenstein and Blanksma succeeded in synthesizing crystalline L-ribose[15] and later D-ribose.[16] The synthetic D-ribose was identical with the crystalline sugar obtained by Levene and Jacobs from the purine nucleosides of yeast nucleic acid.

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[7] M. Bergmann, H. Schotte, and W. Lechinsky, *Ber.* **55,** 158 (1922).

[8] J. C. Sowden, *J. Am. Chem. Soc.* **69,** 1047 (1947).

[9] A. Kossel, *Arch. Anat. u. Physiol., Physiol. Abt.* **1891,** 181.

[10] O. Hammarsten, *Z. physiol. Chem.* **19,** 19 (1894).

[11] P. A. Levene and W. A. Jacobs, *Ber.* **41,** 2703 (1908).

[12] P. A. Levene and W. A. Jacobs, *Ber.* **42,** 1198 (1909); **44,** 746 (1911).

Simultaneous hydrolysis and oxidation of cytidine with hydrobromic acid and bromine affords bromouracil and D-ribonic acid.[17] Since cytidine is readily transformed into uridine by deamination,[18] it follows that the pyrimidine nucleosides of yeast nucleic acid also have D-ribose as the sugar component. Using benzimidazole derivatives Gulland *et al.*[19-21] have restudied recently the problem of the identification of D-ribose in yeast nucleic acid. In the naturally occurring ribosyl-purines and -pyrimidines the sugar is present in the furanose form and is glycosidically linked in the β-configuration to position 9′ of the purine bases or to position 3′ of the pyrimidine bases. (Compare some reviews summarizing the evidence leading to these conclusions.[22, 23]) These conclusions, initially based on analytical experiments, have been confirmed by synthesis studies[3, 24-26] and to some extent by X-ray investigations.[27-29] In nucleic acids, hydroxyl groups in the D-ribose portion of the molecule are concerned in phosphate ester internucleotide linkages. A discussion of these linkages forms the subject of Chapter 12.

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[13] O. Ruff and G. Ollendorf, *Ber.* **32,** 3234 (1899).

[14] E. Fischer and O. Piloty, *Ber.* **24,** 4214 (1891).

[15] W. Alberda van Ekenstein and J. J. Blanksma, *Chem. Weekblad* **6,** 373 (1909).

[16] W. Alberda van Ekenstein and J. J. Blanksma, *Chem. Weekblad* **10,** 664 (1913).

[17] P. A. Levene and F. B. La Forge, *Ber.* **45,** 608 (1912).

[18] P. A. Levene and W. A. Jacobs, *Ber,* **43,** 3150 (1910).

[19] G. R. Barker and J. M. Gulland, *J. Chem. Soc.* **1943,** 625.

[20] G. R. Barker, Kathleen R. Cooke, and J. M. Gulland, *J. Chem. Soc.* **1944,** 339.

[21] G. R. Barker, Kathleen R. Farrar, and J. M. Gulland, *J. Chem. Soc.* **1947,** 21.

[22] R. S. Tipson, *Advances in Carbohydrate Chem.* **1,** 193 (1945).

[23] D. O. Jordan, *Ann. Rev. Biochem.* **21,** 209 (1952).

[24] A. R. Todd, *J. Chem. Soc.* **1946,** 647; *Harvey Lectures, Ser.* **XX,** 1-20, 1951-52.

[25] J. Baddiley, *Roy. Inst. Chem. (London), Lectures, Monographs Repts.* **No. 3,** 1-17 (1950).

[26] A. M. Michelson, *J. Cellular Comp. Physiol.* **38,** Suppl. 1, 11 (1951).

[27] S. Furberg, *Nature* **164,** 22 (1949).

[28] S. Furberg, *Acta Cryst.* **3,** 325 (1950).

[29] S. Furberg, *Acta Chem. Scand.* **4,** 751 (1950).

In addition to its occurrence as a component of ribonucleic acid and of nucleotides like adenosine di- and triphosphate, D-ribose has been found in combination with uric acid in the blood[30] and united with 2-hydroxy-6-aminopurine (isoguanine) in the croton bean (*Croton tiglium* L.).[31, 32] 2-Hydroxy-6-aminopurine-D-riboside (sometimes called crotonoside) can be isolated from the croton seed by extraction with methanol, and Spies and Drake[33] succeeded in obtaining from it crystalline D-ribose. The pentose also occurs in certain vitamins and coenzymes. Acidic degradation of vitamin B_{12} results in the formation of 1-(α-D-ribofuranosyl)-5,6-dimethylbenzimidazole,[34-38] and it has been shown that the ribofuranose moiety of the vitamin is phosphorylated at either C-2 or C-3.[38] Coenzyme I (diphosphopyridine nucleotide) and coenzyme II (triphosphopyridine nucleotide) isolated by Euler *et al.*[39-41] and by Warburg *et al.*[42] from yeast and red blood cells, respectively, coenzyme A[43] and the coenzyme of "galactowaldenase"—uridine diphosphate glucose[44]—all have substituted ribose components as part of their molecules. (See also Chapter 4 and Schlenk[45] and Kornberg and Pricer[46] for further details.) Leloir[2] has suggested that ribose-1,5-diphosphate is probably a coenzyme for the enzyme responsible for the conversion of ribose-1-phosphate to ribose-5-phosphate. Over a quarter of a century ago Winter[47] obtained a substance from certain animal

[30] Alice R. Davis, Eleanor B. Newton, and S. R. Benedict, *J. Biol. Chem.* **54,** 595 (1922)

[31] E. Cherbuliez and K. Bernhard, *Helv. Chim. Acta* **15,** 464 (1932).

[32] E. Cherbuliez and K. Bernhard, *Helv. Chim. Acta* **15,** 978 (1932).

[33] J. R. Spies and N. L. Drake, *J. Am. Chem. Soc.* **57,** 774 (1935).

[34] N. G. Brink, F. W. Holly, C. H. Shunk, Elizabeth W. Peel, J. J. Cahill, and K. Folkers, *J. Am. Chem. Soc.* **72,** 1866 (1950).

[35] N. G. Brink and K. Folkers, *J. Am. Chem. Soc.* **71,** 2951 (1949); **72,** 4442 (1950).

[36] E. R. Holiday and V. Petrow, *J. Pharm. and Pharmacol.* **1,** 734 (1949).

[37] G. R. Beaven, E. R. Holiday, E. A. Johnson, B. Ellis, P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm. and Pharmacol.* **1,** 957 (1949).

[38] J. G. Buchanan, A. W. Johnson, J. A. Mills, and A. R. Todd, *Chemistry & Industry* **1950,** 426; *J. Chem. Soc.* **1950,** 2845.

[39] H. v. Euler, H. Albers, and F. Schlenk, *Z. physiol. Chem.* **237,** 1 (1935).

[40] H. v. Euler, and F. Schlenk, *Z. physiol. Chem.* **246,** 64 (1937).

[41] H. v Euler, P. Karrer, and E. Usteri, *Helv. Chim. Acta* **25,** 323 (1942).

[42] O. Warburg, W. Christian, and A. Griese, *Biochem. Z.* **282,** 157 (1935).

[43] J. Baddiley, E. M. Thain, G. D. Novelli, and F. Lipmann, *Nature* **171,** 76 (1953).

[44] R. Caputto, L. F. Leloir, C. E. Cardini, and A. C. Paladini, *J. Biol. Chem.* **184,** 333 (1950).

[45] F. Schlenk, *J. Biol. Chem.* **146,** 619 (1942).

[46] A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.* **186,** 557 (1950).

[47] L. B. Winter, *Biochem. J.* **21,** 467 (1927).

tissues which he considered to be an ethyl riboside, but confirmation of t
discovery has not been reported. Very recently, however, it has been claim
that the browning occurring in moist fish flesh on exposure to high tempe
tures is due to reactions of the Maillard type[48] and that the sugar contr
uting the aldehyde groups is D-ribose.[49]

2-Deoxy-D-ribose is the only 2-deoxypentose believed to occur in nucl
acids. Chargaff and his co-workers[50, 51] have examined by chromatograp
techniques a range of nucleic acids isolated from animal, plant and b
terial sources and have found no trace of any other deoxysugar. In nucl
acids 2-deoxy-D-ribose is linked glycosidically to either a purine or p
rimidine base. As is the case with ribose, the purine bases are adenine a
guanine. The pyrimidine bases found united to the deoxypentose a
cytosine and thymine (in ribonucleic acids uracil replaces this latter ba
and in some cases 5-methylcytosine in small amounts. Although not
normal component of deoxypentose nucleic acids, the deoxyriboside
uracil has been obtained from an enzymic hydrolysis of a commerc
sample of herring sperm deoxypentose nucleic acid.[52] It is considered
have been formed by an enzyme (cytosine deoxyriboside deaminase
introduced into the digest by bacterial contamination. In these nucleosid
2-deoxy-D-ribose is linked to position 9′ of the purine bases or to positi
3′ of the pyrimidine bases.[54] There is no conclusive evidence concerni
the stereochemical disposition of the deoxyribose-base linkage, althou
Furberg[29] considers that deoxyribonucleotides may have the same sha
as ribonucleotides, in which he demonstrated by X-ray analysis that t
linkage was of the β-D-type. Hammarsten and colleagues[55] have present
evidence from biochemical experiments which can be interpreted[56]
indicating a β-D-configuration for the sugar-base linkage in deoxyribonucle
tides. Until recently, the lactol ring structure of 2-deoxy-D-ribose in nucle
acid had been proved conclusively only for the thymidine nucleoside co
ponent and in this case it was furanose in form.[57] Subsequently Brow
and Lythgoe[58] by application of the periodate oxidation procedure to t

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[48] H. L. A. Tarr, *J. Fisheries Research Board Can.* **8,** 74 (1950).

[49] H. L. A. Tarr, *Nature* **171,** 344 (1953).

[50] E. Chargaff, E. Vischer, R. Doniger, Charlotte Green, and F. Misani, *J. Bi Chem.* **177,** 405 (1949).

[51] E. Chargaff and Rakoma Lipshitz, *J. Am. Chem. Soc.* **75,** 3658 (1953).

[52] C. A. Dekker and A. R. Todd, *Nature* **166,** 557 (1950).

[53] T. P. Wang, H. Z. Sable, and J. O. Lampen, *J. Biol. Chem.* **184,** 17 (1950).

[54] J. M. Gulland and L. F. Story, *J. Chem. Soc.* **1938,** 259, 692.

[55] E. Hammarsten, P. Reichard, and E. Saluste, *J. Biol. Chem.* **183,** 105 (1950).

[56] J. Baddiley, *Ann. Rev. Biochem.* **20,** 172 (1951).

[57] P. A. Levene and R. S. Tipson, *Science* **81,** 98 (1935); *J. Biol. Chem.* **109,** 623 (193

[58] D. M. Brown and B. Lythgoe, *J. Chem. Soc.* **1950,** 1990.

-deoxyribosides of guanine, hypoxanthine, cytosine and thymine, afforded roof of the presence of a furanose sugar in each compound.

Besides 2-deoxy D-ribose, other derivatives of D-ribose occur naturally. 3-Amino--ribose has been shown to be one of the products of hydrolysis of Puromycin,[59, 60] d 5-thiomethyl D-ribose occurs in the adenylthiomethylpentose[61] which has been olated from yeast, crude vitamin B_1 or crude cozymase. [Cf. *Baddiley*, Chapter 4.] eduction products of D-ribose are also found in Nature. For example ribitol (adoni-ol) is isolable from *Adonis vernalis* L.[62, 63] and the reduced ribityl residue forms part f the riboflavin (vitamin B_2, 6,7-dimethyl-9-(D-ribityl)isoalloxazine) molecule nd of the coenzyme D-riboflavin-5′-phosphate (flavin mononucleotide). Despite the bsence of the ring structure of the sugar in these derivatives of ribitol, the stereo-hemistry of the sugar residue is of great biological importance.[64] Synthesis of analo-ous flavins containing other sugar residues gives rise to substances of quite different iological activity. Flavin adenine dinucleotide, a mixed diphosphate ester of ribo-avin and adenosine, contains both ribofuranose and ribityl residues in its molecule. his substance, which is the coenzyme chiefly involved in transporting electrons from he reduced pyridine nucleotides to cytochrome, has been demonstrated in blood ells,[65] liver, heart,[66] brain, kidney, intestine[67] and muscle.[68] It is probably a matter f some significance that, in naturally occurring derivatives of ribose, the sugar is resent in the furanose form.

III. Chemistry of Ribose

1. Preparation

The first synthesis of ribose was achieved by Fischer and Piloty[14] in 1891 by epimerization of L-arabonic acid to L-ribonic acid and subsequent reduction of its lactone to syrupy L-ribose. Since that time many attempts have been made to convert derivatives of glucose and arabinose into ribose. Blanksma and Alberda van Ekenstein[69] repeated the work of Fischer and Piloty and obtained a syrupy product which was contaminated with ribitol. Subsequently, the crude ribose was purified through its *p*-bromophenylhydrazone and crystalline L-ribose was obtained for the first time.[15] The same workers repeated the synthesis in the D-series and by pyridine treatment succeeded in converting D-arabonic acid (IV) into D-ribonic

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[59] C. W. Waller, P. W. Fryth, B. L. Hutchings, and J. H. Williams, *J. Am. Chem. Soc.* **75,** 2025 (1953).

[60] B. R. Baker and R. E. Schaub, *J. Am. Chem. Soc.* **75,** 3864 (1953).

[61] J. A. Mandel and E. K. Dunham, *J. Biol. Chem.* **11,** 85 (1912).

[62] W. V. Podwissotzky, *Arch. pharm.* **227,** 141 (1889); quoted by R. W. Jeanloz and H. G. Fletcher, Jr., *Advances in Carbohydrate Chem.* **6,** 145 (1951).

[63] E. Merck, *Arch. pharm.* **231,** 129 (1893).

[64] E. L. Hirst, *J. Chem. Soc.* **1949,** 522.

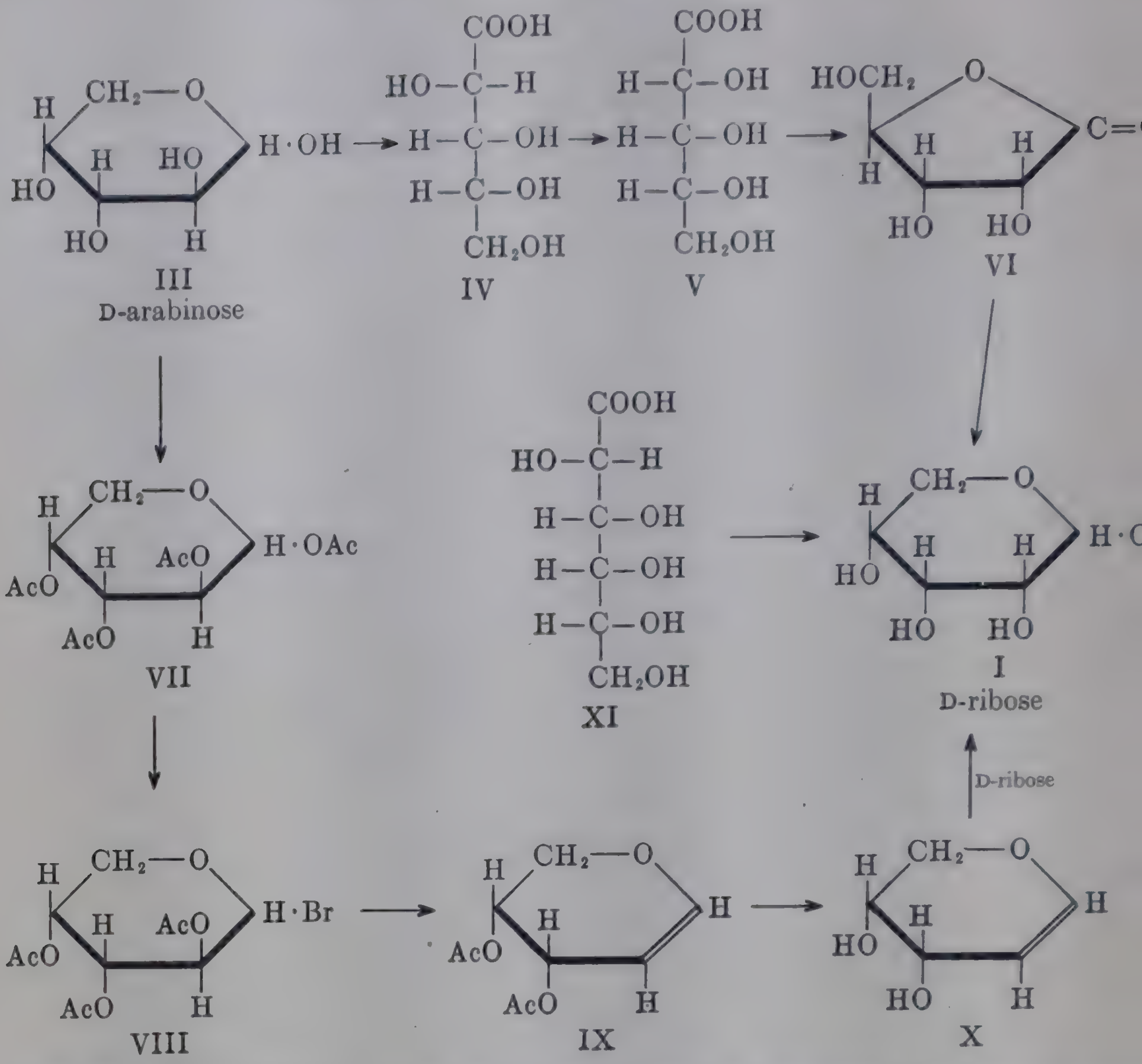
[65] J. R. Klein and H. I. Kohn, *J. Biol. Chem.* **136,** 177 (1940).

[66] S. Ochoa and R. J. Rossiter, *Biochem. J.* **33,** 2008 (1939).

[67] A. V. Trufanov, *Biokhimiya* **6,** 301 (1941).

[68] A. V. Trufanov, *Biokhimiya* **7,** 188 (1942).

[69] J. J. Blanksma and W. Alberda van Ekenstein, *Chem. Weekblad* **5,** 777 (1908).



acid (V). Thereafter by reduction with sodium amalgam and purificati
via the p-bromophenylhydrazone, a crystalline sample of D-ribose (
was obtained.[16] This method of synthesis was reinvestigated thoroughly
Steiger[70] in 1936. D-Arabinose (III) was oxidized electrolytically[71] to D-ar
bonic acid (IV). IV was partly epimerized through the action of boili
aqueous pyridine and D-ribonic acid (V) was isolated as its cadmium sa
and converted into D-ribonolactone (VI). Controlled reduction of t
lactone with 2.5% sodium amalgam gave D-ribose (I). More product cou
be obtained from the mother liquors as the p-bromophenylhydrazone.
this method D-ribose was obtained from D-arabinose in 17% overall yiel

Alberda van Ekenstein and Blanksma[72] claimed that L-arabinose may
partly converted directly to L-ribose through the Lobry de Bruyn rearrang
ment, but the process has no preparative value. Austin *et al.*[73] failed
confirm the findings of van Ekenstein and Blanksma,[72] since they we

[70] Marguerite Steiger, *Helv. Chim. Acta* **19**, 189 (1936).

[71] H. S. Isbell and Harriet L. Frush, *J. Research Natl. Bur. Standards* **6**, 1145 (193

[72] W. Alberda van Ekenstein and J. J. Blanksma, *Chem. Weekblad* **10**, 213 (1913).

[73] W. C. Austin, C. J. Smalley, and M. I. Sankstone, *J. Am. Chem. Soc.* **54**, 19
(1932).

nable to obtain crystalline ribose, its *p*-bromophenylhydrazone or crystalline derivatives (i.e., phenylhydrazide or cadmium salt) of ribonic acid from syrups obtained by treating D- or L-arabinose with alkali. Alberda van Ekenstein and Blanksma heated L-arabinose in *N* sodium hydroxide solution and then oxidized the product to a mixture of L-arabonic and L-ribonic acids which were separated by fractional crystallization of their phenylhydrazides. Except that they used calcium hydroxide (0.04 *N*) as alkali, Austin *et al.* employed the same procedure. Explanations of the different results reported by these two groups of workers have been offered in terms of different temperatures for epimerization, nature of the alkali, and concentration effects, but these are not entirely convincing.

Ribose has been obtained from arabinose by Gehrke and Aichner[74] by application of a method originally developed by Bergmann and Schotte[75] during their researches with glucal. D-Arabinose (III) was converted via its tetraacetate (VII) and 2,3,4-tri-*O*-acetyl-D-arabinopyranosyl bromide (VIII) into 3,4-di-*O*-acetyl-D-arabinal (IX). (This compound can also be named 3,4-di-*O*-acetyl-D-ribal.) After deacetylation, the product (X, D-arabinal = D-ribal) in chloroform solution was hydroxylated at 0° with perbenzoic acid to give a mixture of D-arabinose (III) and D-ribose (I), with the latter predominating. The ribose was purified via its crystalline benzylphenylhydrazone. The sequence of reactions was also carried out using L-arabinose as the initial material and in this case the syrupy product was converted by oxidation into crystalline L-ribonolactone. The method was extended and improved by Austin and Humoller[76] (cf. Neker and Lewis[77] for related work), purification of the product being achieved by direct crystallization and through formation of the *p*-bromophenylhydrazone. It was shown that treatment of L-arabinal with perbenzoic acid in ethyl acetate at 0° yields 5 parts of L-ribose to 1 part of L-arabinose and the overall yield of crystalline ribose, based on the arabinose used, was nearly 10% of theoretical. Workers in other laboratories[78, 79] have also employed this method to prepare ribose and frequently obtained the arabinose needed as starting material from calcium D-gluconate by descent of the sugar series. Hudson and Richtmyer[80] have used this latter method to convert the calcium salt of D-altronic acid (XI) (for the preparation of calcium D-altronate see Richtmyer[81]) into D-ribose (I) by the improve Ruff[83] degradation method for converting hexonic acids into pentoses.

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[74] M. Gehrke and F. X. Aichner, *Ber.* **60,** 918 (1927).

[75] M. Bergmann and H. Schotte, *Ber.* **54,** 450 (1921).

[76] W. C. Austin and F. C. Humoller, *J. Am. Chem. Soc.* **54,** 4749 (1932); **56,** 1152 (1934).

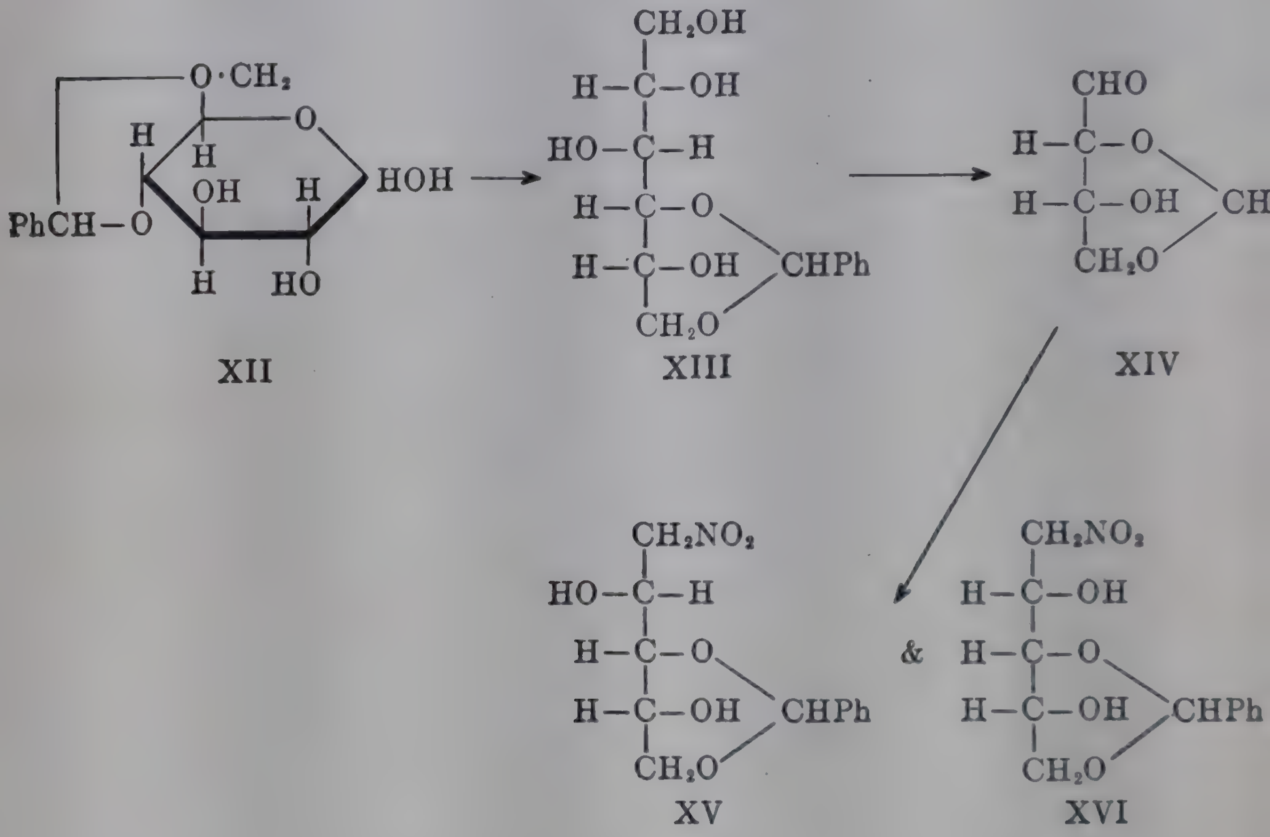
[77] H. T. Neker and W. L. Lewis, *J. Am. Chem. Soc.* **53,** 4411 (1931).

[78] R. Kuhn, K. Reinemund, F. Weygand, and R. Ströbele, *Ber.* **68,** 1765 (1935).

[79] P. Karrer, B. Becker, F. Benz, P. Frei, H. Salomon, and K. Schöpp, *Helv. Chim. Acta* **18,** 1435 (1935).

[80] C. S. Hudson and N. K. Richtmyer, U. S. Pat. 2,162,721 (July 20, 1939).

Recently Sowden[5] has introduced a method (cf. Fischer and Sowden whereby D-ribose can be obtained from D-glucose. 4,6-O-Benzylidene- glucose (XII) was reduced catalytically to 4,6-O-benzylidene-D-glucit (XIII) which on oxidation with sodium metaperiodate yielded 2,4- benzylidene-D-erythrose (XIV). Condensation of XIV with nitrometha gave a mixture of epimeric, crystalline substituted C-nitro-alcohols, namel 3,5-O-benzylidene-1-deoxy-1-nitro-D-arabitol (XV) and 3,5-O-benzyliden 1-deoxy-1-nitro-D-ribitol (XVI).



Separation of XV and XVI was achieved by virtue of a remarkable di ference in their solubility in chloroform: XVI was very soluble in this so vent. Hydrolysis of XVI afforded amorphous 1-deoxy-1-nitro-D-ribit which in the form of its sodium salt was decomposed directly to D-ribos The pentose was isolated as the benzylphenylhydrazone. The purificati of syrupy samples of ribose can be achieved by the tedious procedure forming the pure crystalline p-bromophenylhydrazone[14, 16, 70] or benzy phenylhydrazone[5, 74] or p-toluenesulfonylhydrazone.[85] A more convenie method has been introduced by Lee and Berger and their co-workers.[86, 87] The technique is based upon the fact that arylamine-*N*-ribosides (e.g., *N*-phenyl-D-ribosylamine) prepared by dissolving the crude ribose in an aqueous-alcoholic solution of an arylamine, form "complex salts" with the soluble salts of alkali metals. (The sugar-*N*-glycoside forms a loose combination with the inorganic salt.) The arylamine-*N*-riboside may be extracted from this complex with dioxane and decomposed by boiling water containing a trace (0.25 %) of acetic acid. Alternatively, aqueous hydrolysis of the arylamine-*N*-riboside, catalyzed by bases, or in the presence of aldehydes such as benzaldehyde, regenerates the original amine and D-ribose.[88, 89] After removal of the arylamine, either by steam distillation or by forming the benzylidene derivative, pure crystalline ribose may be obtained in yields of 70–90 %. Triacylribose derivatives can be prepared by this procedure from arylamine *N*-acylated ribosides. Thus, pure 2,3,4-tri-*O*-acetyl-D-ribose was obtained by hydrolysis of the 2,3,4-tri-*O*-acetyl-*N*-phenyl-α-D-ribosylamine[90] which resulted from the anilination of crude 2,3,4-tri-*O*-acetyl-D-ribose and purification of the "anilide" via a "complex salt."

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[81] N. K. Richtmyer, *Advances in Carbohydrate Chem.* **1**, 37 (1945).

[82] R. C. Hockett and C. S. Hudson, *J. Am. Chem. Soc.* **56**, 1632 (1934).

[83] O. Ruff, *Ber.* **32**, 550 (1899); **35**, 2360 (1902).

[84] J. C. Sowden and H. O. L. Fischer, *J. Am. Chem. Soc.* **69**, 1048 (1947).

[85] D. G. Easterby, L. Hough, and J. K. N. Jones, *J. Chem. Soc.* **1951**, 3416.

The preparation of D-ribose from yeast nucleic acid has been extensively investigated. Levene and Clark[91] developed a method involving hydrolysis of the nucleic acid with ammonia at an elevated temperature and pressure. From the resultant mixture of nucleosides, adenosine and guanosine were separated, purified and subjected to further hydrolysis to give D-ribose. Levene[92] described a preparation of crystalline D-ribose by acidic hydrolysis (0.05 *N* sulfuric acid at the reflux temperature for 2 hours) of pure ash-free guanosine. It was considered essential by Levene to work with a colorless hydrolysate, if pure ribose is required. For the hydrolysis of nucleic acid, Phelps[93] replaced ammonia by magnesia and heated the mixture at 145° for 4 hours. Magnesium-containing phosphates are removed by filtration and then guanosine precipitates directly and adenosine is recovered from the mother liquors as the picrate. Acidic hydrolysis of either guanosine or adenosine picrate results in the liberation of D-ribose. (See also Bates and associates[94] for preparative details of D-ribose from nucleic acid.)

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[86] J. Lee, U. V. Solmssen, and L. Berger, U. S. Pat. 2,384,103 (Sept. 4, 1945).

[87] L. Berger and J. Lee, *J. Org. Chem.* **11,** 84 (1946).

[88] L. Berger, U. V. Solmssen, F. Leonard, Ed. Wenis, and J. Lee, *J. Org. Chem.* **11,** 91 (1946).

[89] J. Lee, E. Fells, and L. Berger, U. S. Pat. 2.383,977 (Sept. 4, 1945).

[90] J. Lee and L. Berger, U. S. Pat. 2.384,104 (Sept. 4, 1945).

[91] P. A. Levene and E. P. Clark, *J. Biol. Chem.* **46,** 19 (1921).

[92] P. A. Levene, *J. Biol. Chem.* **108,** 419 (1935).

[93] F. P. Phelps, U. S. Pat. 2,152,662 (April 4, 1939).

[94] F. J. Bates and associates, "Polarimetry, Saccharimetry and the Sugars," *Natl. Bur. Standards (U.S.) Circ.* **C440,** 476 (1942).

Hydrolysis of aqueous solutions of yeast nucleic acid can be accomplished by the agency of enzymic preparations from sweet almonds, lucerne seeds or germinated peas.[95] Good yields of guanosine and adenosine are obtained, and acidic hydrolysis of the former nucleoside yields D-ribose.[95, 96] Details for the isolation of D-ribose from yeast nucleic acid have been patented by Laufer and Charney;[97] the method aims essentially at facilitating the separation of nucleosides from a hydrolysate of the nucleic acid.

Addition of cuprous ions results in precipitation of the purine nucleosides as cuprous salts. Acidic hydrolysis of the salts results in the formation of D-ribose and the insoluble cuprous salts of adenine and guanine. Alternatively, the nucleic acid is hydrolyzed to a mixture of nucleotides which are converted into their cuprous salts by addition of copper sulfate and sodium bisulfite. The pyrimidine nucleotides remain in solution, but the purine nucleotides are precipitated. After separation and washing these purine nucleotides are subjected to hydrolysis with sulfuric acid. Insoluble cuprous salts of adenine and guanine are removed from the hydrolysate by filtration, leaving a solution containing D-ribose and sulfuric and phosphoric acids. Addition to the solution of an alkaline earth hydroxide followed by filtration gives a solution of practically pure D-ribose.[98] Nowadays it is more usual to separate nucleoside and nucleotide mixtures by ion-exchange resin chromatography.[99] [Cf. Cohn, Chapter 6.]

2. IDENTIFICATION AND ESTIMATION

A tentative identification of ribose may be achieved from the usual tests for a reducing sugar and specific tests for pentoses. Determinations on crystalline samples of the melting point, specific rotation and optical crystallographic properties will serve to differentiate between the various pentoses. Of the derivatives which can be prepared for characterization, substituted hydrazones have been preferred. At various times the *p*-bromophenylhydrazone,[12, 14, 15, 33, 70, 76, 79, 100-103] the benzylphenylhydrazone,[12, 74] the *p*-toluenesulfonylhydrazone[85, 104] and to a lesser extent the diphenylmethane-dimethyl-dihydrazone[105] have been used for this purpose. Ribose may also be characterized by preparing 2-(*ribo*-tetrahydroxybutyl)benzimidazole (ribobenzimidazole)[20, 21, 106, 107] from ribonic acid and *o*-phenylenediamine. This latter method of characterization is not entirely unequivocal since Dimler and Link[107] have demonstrated that the conversion of ribose to ribonic acid by oxidation with alkaline hypoiodite results in slight (5%) epimerization and so the product is contaminated with arabonic acid. Epimerization at this stage may be avoided by using the improved procedure for preparing aldonic acids described by Hudson and Isbell,[108] but there is a further risk of epimerization during the condensation of the aldonic acid with *o*-phenylenediamine, particularly if there is insufficient acid present.[19-21] (For a review of substituted benzimidazoles derived from sugar acids see Richtmyer.[109])

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[95] H. Bredereck and G. Rothe, *Ber.* **71,** 408 (1938).

[96] H. Bredereck, M. Köthnig, and Eva Berger, *Ber.* **73,** 956 (1940).

[97] L. Laufer and J. Charney, U. S. Pat. 2,379,913 (July 10, 1945).

[98] L. Laufer and J. Charney, U. S. Pat. 2,379,914 (1945).

[99] W. E. Cohn, *Science* **109,** 377 (1949); *J. Am. Chem. Soc.* **71,** 2275 (1949); *J. Am. Chem. Soc.* **72,** 1471 (1950).

[100] W. Alberda van Ekenstein and J. J. Blanksma, *Chem. Weekblad* **11,** 182 (1914); *Brit. Abstr.* **106** (i), 388 (1914).

[101] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **115,** 731 (1936).

[102] C. W. Klingensmith and W. L. Evans, *J. Am. Chem. Soc.* **61,** 3012 (1939).

[103] P. A. Levene and W. A. Jacobs, *Ber.* **42,** 3247 (1909).

[104] B. Helferich and H. Schirp, *Chem. Ber.* **86,** 547 (1953).

The β-tetraacetate[33, 110] and β-tetrabenzoate[111] and various mercaptals[112, 113] have frequently been used. Zinner[114] recommends the di-*n*-propyl and di-isobutyl mercaptals for the characterization of ribose. Chromatographic methods have been used to identify ribose in mixtures of sugars and to separate it from such mixtures. [Compare Chapters 6 and 7.] Various reagents have been used to demonstrate the position of the sugar spots on paper chromatograms: aniline hydrogen phthalate[115, 116] in moist butanol gives a bright red color with pentoses which may be differentiated from aldohexoses, uronic acids and deoxysugars as these give various shades of green and brown. It is possible with this reagent to detect 1 μg. of ribose. Benzidine can be used as a developing agent[117] and pentoses are shown as chocolate-brown colored spots on a chromatogram. Jones *et al.*[118] have described experiences using various acidic spray reagents, as for example, *p*-anisidine hydrochloride and aniline trichloroacetate and have reported R_F values for ribose. Potassium permanganate with sodium carbonate has been used[119] successfully for indicating the position of D-ribose on paper chromatograms.

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[105] J. v. Braun, *Ber.* **46,** 3949 (1913).

[106] N. K. Richtmyer and C. S. Hudson, *J. Am. Chem. Soc.* **64,** 1612 (1942).

[107] R. J. Dimler and K. P. Link, *J. Biol. Chem.* **150,** 345 (1943).

[108] C. S. Hudson and H. S. Isbell, *J. Research Natl. Bur. Standards* **3,** 57 (1929).

[109] N. K. Richtmyer, *Advances in Carbohydrate Chem.* **6,** 175 (1951).

[110] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **92,** 109 (1931).

[111] R. Jeanloz, H. G. Fletcher, Jr., and C. S. Hudson, *J. Am. Chem. Soc.* **70,** 4052 (1948).

[112] E. Hardegger, E. Schreier, and Z. El Heweihi, *Helv. Chim. Acta* **33,** 1159 (1950).

[113] H. Zinner, *Chem. Ber.* **86,** 496 (1953).

[114] (a) H. Zinner, *Chem. Ber.* **83,** 275 (1950); (b) see also *Chem. Ber.* **86,** 495 (1953).

[115] S. M. Partridge, *Nature* **164,** 443 (1949).

[116] Judith Blass, M. Macheboeuf, and G. Nunez, *Bull. soc. chim. biol.* **32,** 130 (1950).

[117] R. H. Horrocks, *Nature* **164,** 444 (1949).

[118] L. Hough, J. K. N. Jones, and W. H. Wadman, *J. Chem. Soc.* **1950,** 1702.

Ribose may be determined by conversion into furfuraldehyde which may be estimated as the phloroglucide[120, 121] by colorimetric methods[122, 123] or by separation on the paper-partition chromatogram and subsequent estimation by sodium metaperiodate.[124, 125] Under standard conditions the formation of crystalline D-ribose *p*-toluenesulfonylhydrazone is quantitative and may be used for the determination of D-ribose.[85] Solutions of the pentose (1–4 %) in methanol may be determined with an accuracy of ±1 %.

3. Properties and Derivatives

a. Physical Properties

Ribose (D-, L- and DL-forms) crystallizes without water of hydration. Values reported by various workers for the melting point and specific rotation are listed in the Appendix (Table VII) (p. 65). The refractive index and optical crystallographic properties of the D-isomer have been described,[126] and Ellinghaus[127] has reported the heat of combustion for the sugar. From results of a polarographic study of D-ribose, Cantor and Peniston[128] concluded that in aqueous solution 8.5 to 30 % of this sugar is present in the aldehyde form. (The corresponding value for arabinose is 0.13 to 0.4 %.) The mutarotation of D- and L-ribose in aqueous solution at 1° was studied by Isbell and his colleagues,[129] and was shown to be complex. The mutarotation takes place rapidly and the direction of the change reverses after a few minutes so that the initial and final rotations are not very different. On account of the rapidity with which the reaction takes place, the mutarotational changes are best observed at low temperatures. It was concluded that in solution, equilibria probably exist involving both pyranose and furanose forms. The initial mutarotational change of D-ribose suggests that in the usual crystalline form it exists as the β-anomer. Bredereck *et al.*[96] examined the mutarotation of D-ribose (at 20°) and 5-*O*-trityl-D-ribose (at 3°) in pyridine solution. Whereas the mutarotation of D-ribose

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[119] E. Pacsu, T. P. Mora, and P. W. Kent, *Science* **110,** 446 (1949).

[120] A. W. Schorger, *Ind. Eng. Chem.* **15,** 742 (1923).

[121] C. Dorée, "Methods of Cellulose Chemistry," p. 381. Chapman & Hall, London, 1947.

[122] Sonia Dunstan and A. E. Gillam, *J. Chem. Soc.* **1949,** S. 140.

[123] G. R. Barker, *J. Chem. Soc.* **1950,** 1636.

[124] E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.* **1949,** 1659.

[125] E. L. Hirst, L. Hough, and J. K. N. Jones, *J. Chem. Soc.* **1949,** 928.

[126] G. T. Keenan, *J. Wash. Acad. Sci.* **16,** 433 (1926).

[127] J. Ellinghaus, *Z. physiol. Chem.* **164,** 308 (1927).

[128] S. M. Cantor and Q. P. Peniston, *J. Am. Chem. Soc.* **62,** 2113 (1940).

[129] F. P. Phelps, H. S. Isbell, and W. Pigman, *J. Am. Chem. Soc.* **56,** 747 (1934).

TABLE I

OXIDATION OF RIBOSE WITH BROMINE

Sugar	Av. value of velocity const. $\times 10^3$	Relative reaction rates $K_{\text{sugar}}/K_{\alpha\text{-D-glucose}}$	Ratio of rates for α- and β-isomers K_β/K_α	Mutarotation constant at 0.2° ± 0.2°C.	
				$m_1 \times 10^3$	$m_2 \times 10^3$
D-Ribose (crystalline)	196	6.1	5.2	—	—
β-D-Ribose (from equilibrated solution)	1010	32		—	—
L-Ribose (crystalline)	195	6.1	7.5	6.87	54.0
β-L-Ribose from equilibrated solution)	1456	45.5		—	—

TABLE II

R_F VALUES (CORRECTED TO 20°) OF D-RIBOSE ON WHATMAN NO. 1 FILTER PAPER

Solvent[a]	Additions[a]	R_F
Phenol	NH_3 (1% wt./vol.) HCN	0.59
s-Collidine	None	0.56
n-BuOH (40%) Acetic acid (10%) Water (50%)	None	0.31
n-BuOH (40%) Ethanol (10%) Water (50%)	NH_3 (1%)	0.285
n-BuOH (45%) Ethanol (5%) Water (49%)	NH_3 (1% wt./vol.)	0.210
n-BuOH	NH_3 (1% wt./vol.)	0.180
Isobutyric acid	None	0.220
Methyl ethyl ketone	NH_3 (1% wt./vol.)	0.165

[a] Unless otherwise stated, all percentages are on a vol./vol. basis.

in this solvent is complex, that of the 5-trityl derivative, which cannot exist in a pyranose form, is of the normal first-order type of reaction. The explanation forwarded for the anomalous results with D-ribose was based on furanose-pyranose interconversions. The bromine oxidation of ribose equilibrated in aqueous solution has been investigated.[129, 130] Initial rapid oxidation is followed by a decrease in the reaction rate, a change ascribed to the presence of a small quanity of some form which is oxidized more readily than the crystalline form of the sugar. Table I illustrates the results obtained.

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[130] H. S. Isbell and W. Pigman, *J. Research Natl. Bur. Standards* **18**, 141 (1937).

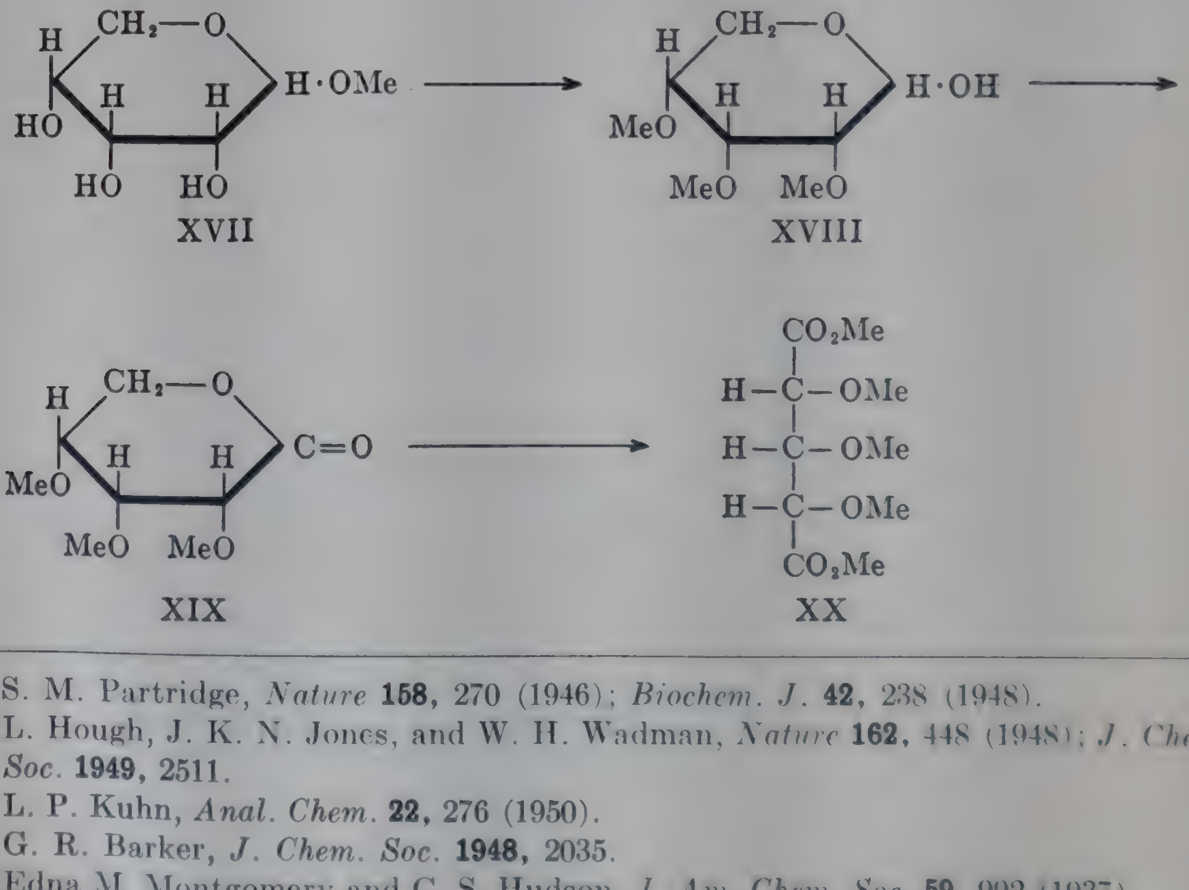
The chromatographic behavior of ribose has been extensively studied and, as stated, the technique of partition chromatography has been used to identify the sugar, particularly when it is present in small quantities in admixture with other substances. Partridge[131] has reported R_F values for D-ribose in a variety of solvent mixtures (see Table II) and has shown that as little as 30 μg. of the sugar can be handled according to his procedure.

Using a column of powdered cellulose, separation of D-ribose from a four-component mixture containing also galactose, rhamnose and arabinose, was achieved successfully by Jones *et al.*[132] Working on a 100–500 mg. scale, crystalline ribose was recovered from this mixture in 94 % yield.

Kuhn[133] has measured the infrared absorption of D-ribose from 8 to 15 microns.

b. *O-Glycosides*

As mentioned previously, Winter[47] claimed to have evidence supporting the existence of two pentose derivatives in animal (i.e., goats) tissues (e.g., liver and muscle), one of which was probably an alkyl(ethyl) D-riboside. Confirmation of the discovery has not yet been reported. Winter considered the possibility that the ethyl riboside might have arisen during the process of tissue extraction, but dismissed the idea, since arabinose failed to yield a glycoside under the conditions used for the isolation. In view of subsequent work by Barker[134] this conclusion was not justified, since ribose rapidly forms a methyl ribofuranoside whereas methyl arabofuranoside forms more slowly.[135]



XVII XVIII

XIX XX

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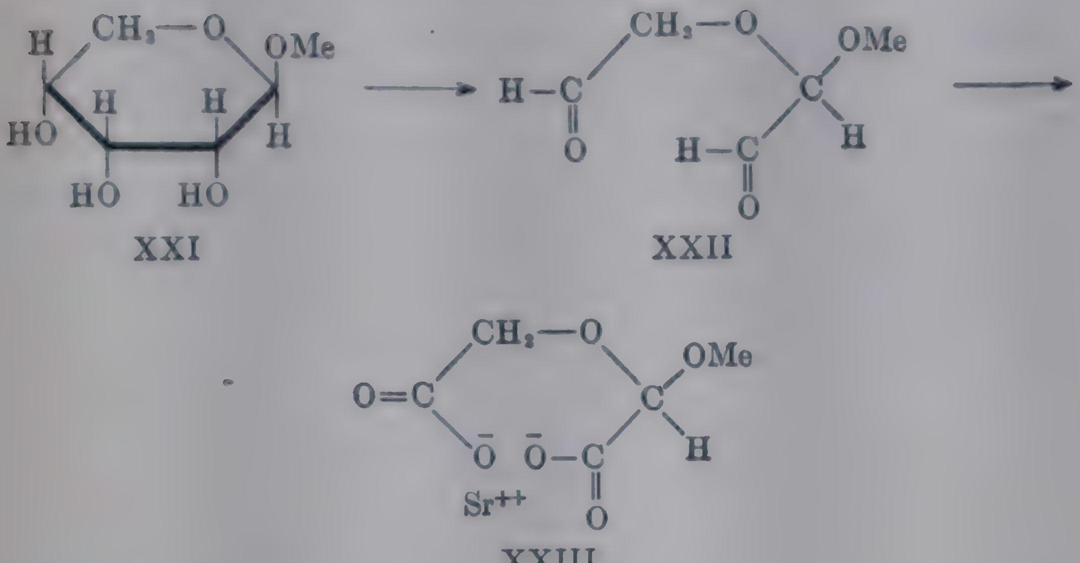
[131] S. M. Partridge, *Nature* **158**, 270 (1946); *Biochem. J.* **42**, 238 (1948).

[132] L. Hough, J. K. N. Jones, and W. H. Wadman, *Nature* **162**, 448 (1948); *J. Chem. Soc.* **1949**, 2511.

[133] L. P. Kuhn, *Anal. Chem.* **22**, 276 (1950).

[134] G. R. Barker, *J. Chem. Soc.* **1948**, 2035.

[135] Edna M. Montgomery and C. S. Hudson, *J. Am. Chem. Soc.* **59**, 992 (1937).


XXI XXII XXIII

Levene and Tipson[110] warmed D-ribose with methanolic hydrogen chloride and obtained a syrupy methyl D-riboside (XVII). Methylation[136] of this glycoside followed by acidic hydrolysis afforded crystalline tri-*O*-methylribose (XVIII). Oxidation of XVIII with bromine yielded a tri-*O*-methylribonolactone (XIX) which hydrolyzed at a rate characteristic for δ(1,5)-lactones. When XVIII was oxidized with nitric acid (d., 1.42) and the product esterified, the diester XX obtained was devoid of optical activity and was in fact methyl *i*-trimethoxyglutarate. It was apparent from this sequence of reactions that XVII had a pyranose ring structure and that XVIII was 2,3,4-tri-*O*-methyl-D-ribose. Soon after publication of the above work, the preparation of a crystalline methyl D-riboside was reported by Minsaas.[137] This glycoside was obtained by treatment of D-ribose with 0.25% methanolic hydrogen chloride, and it exhibited a different specific rotation from that reported by Levene and Tipson[110] for their syrupy product (XVII). The structure of this crystalline compound was subsequently investigated by Jackson and Hudson[138] and it was proved to be methyl β-D-ribopyranoside (XXI). For this work the glycoside was prepared by slight modification of Minsaas' procedure. Oxidation of the methyl riboside with periodic acid or sodium metaperiodate resulted in the consumption of 2.02 moles of oxidant and simultaneous formation of 1 mole of formic acid, thereby indicating that the material was a glycopyranoside. The dialdehyde (XXII) also formed in this oxidation was further oxidized with bromine to a dibasic acid, isolated as its strontium salt (XXIII). The identity of XXIII with the strontium salts obtained when either methyl β-D-arabinopyranoside or methyl β-D-xylopyranoside were subjected to the same treatment[139] demonstrated that XXI had the β-glycosidic configuration. Lattice constants have been calculated[140] for this crystalline glycoside. The crystals were rhombic and values obtained were $a = 5.75 \pm 0.02$ A.; $b = 6.39 \pm 0.02$ A.; $c = 19.90 \pm 0.03$ A.

Barker[134] has shown that the initial product of the action of methanol containing 1% hydrogen chloride on D-ribose is methyl D-ribofuranoside. The structure of this glycoside was established by the classical method of methylation, hydrolysis and oxidation to a lactone, which hydrolyzed at a rate characteristic of a γ(1,4)-lactone.

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[136] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **93,** 623 (1931).

[137] J. Minsaas, *Ann.* **512,** 286 (1934).

[138] E. L. Jackson and C. S. Hudson, *J. Am. Chem. Soc.* **63,** 1229 (1941).

[139] E. L. Jackson and C. S. Hudson, *J. Am. Chem. Soc.* **59,** 994 (1937); **61,** 1530 (1939).

[140] H. Braekken, *Kgl. Norske Videnskab. Selskabs Forh.* **9** 184 (1936).

Hence it was 2,3,5-tri-*O*-methyl-D-ribonolactone rather than the corresponding 2,3,4-derivative, and therefore the original glycoside must have had a furanose structure.

Syntheses of alkyl ribosides by treatment of triacylribopyranosyl halides with alcohols have been frequently reported. Levene and Tipson[110] prepared a crystalline methyl 2,3,4-tri-*O*-acetyl-D-riboside by reacting tri-*O*-acetyl-D-ribopyranosyl bromide with methanol in the presence of silver carbonate. The tri-*O*-acetyl-D-ribopyranosyl bromide was made by acting on crystalline D-ribose 1,2,3,4-tetra-*O*-acetate with hydrogen bromide. The halide derivative was obtained in crystalline form and showed a value of −209.3° in chloroform solution for its specific rotation, and so presumably belongs to the β-series. It is markedly unstable and is reconverted to ribose tetra-*O*-acetate by shaking with silver acetate. The corresponding tri-*O*-acetyl-β-D-ribopyranosyl chloride has been prepared in crystalline form,[141] but the acetylated D-ribofuranosyl halides are very unstable, and although both the chloride[141-143] and the bromide[141, 144] have been handled in various researches, neither has been adequately characterized. The benzoylated D-ribopyranosyl halides apparently are more stable than the acetyl analogues. Jeanloz *et al.*[111] obtained 2,3,4-tri-*O*-benzoyl-D-ribopyranosyl bromide in crystalline form through the action of hydrogen bromide on β-D-ribopyranose 1,2,3,4-tetra-*O*-benzoate in glacial acetic acid. The compound could be stored at 5° indefinitely if kept over calcium chloride and caustic potash. The β-configurational assignment was based on optical rotation measurements, and the pyranose structure follows from the observation that on reaction with methanol in the absence of an acid-acceptor the ribose halide derivative is converted to methyl 2,3,4-tri-*O*-benzoyl-β-D-ribopyranoside,[145] identical with the product obtained by benzoylation of methyl β-D-riboside (XXI) prepared according to Minsaas, the structure of which was established as described above. Since tri-*O*-benzoyl-β-D-ribopyranosyl bromide may be obtained from D-ribose in 68% of the theoretical yield and will react with methanol to give the benzoylated methyl riboside in high yield (88%) and since debenzoylation can be carried out in nearly quantitative yield, Jeanloz and Fletcher[1] claim that this route for the synthesis of alkyl ribopyranosides is rather more attractive than that involving direct condensation of the sugar with an alcohol, particularly as the latter procedure leads to mixtures from which it is often difficult to obtain the required product in crystalline form. Furthermore it is not absolutely necessary to isolate the halogenated sugar in syntheses of alkyl ribosides. Ethyl β-D-ribopyranoside has been prepared by both routes.[145] The pyranose structure was confirmed by periodate titration.

Treatment of 2,3,4-tri-*O*-benzoyl-β-D-ribopyranosyl bromide with methanol in the presence of an acid-acceptor (i.e., silver carbonate) yielded an amorphous mixture from which no crystalline material could be obtained.[145] Reinvestigation of this reaction and also of the reaction between β-D-ribopyranose tetra-*O*-benzoate and hydrogen bromide in glacial acetic acid, by Ness, Fletcher and Hudson,[146] led to the isolation in low yield (5.2%) of a new crystalline tri-*O*-benzoyl-D-ribopyranosyl bromide, isomeric with that previously known. It was dextrorotatory and was considered to be the α-isomer. The new α-isomer could also be obtained in very low yield (1.3%) when tri-*O*-benzoyl-β-D-ribopyranosyl bromide was treated with hydrogen bromide in glacial acetic acid. Most of the starting material (i.e., β-isomer) (96%) could be recovered unchanged.

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[141] H. Zinner, *Chem. Ber.* **83,** 153 (1950).

[142] G. A. Howard, A. C. McLean, G. T. Newbold, F. S. Spring, and A. R. Todd, *J. Chem. Soc.* **1949,** 232.

[143] J. Davoll, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1948,** 967.

[144] G. A. Howard, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1947,** 1052.

[145] R. Jeanloz, H. G. Fletcher, Jr., and C. S. Hudson, *J. Am. Chem. Soc.* **70,** 4055 (1948).

[146] R. K. Ness, H. G. Fletcher, Jr., and C. S. Hudson, *J. Am. Chem. Soc.* **73,** 959 (1951).

Treatment of β-D-ribopyranose tetra-*O*-benzoate with titanium tetrachloride in chloroform solution gives two crystalline products which are considered to be tri-*O*-benzoyl-α- and -β-D-ribopyranosyl chloride. The major product is the β-anomer. Both compounds react with methanol in the absence of an acid-acceptor to give methyl tri-*O*-benzoyl-β-D-ribopyranoside.

Further evidence in favor of the assigned configurations for the triacylribopyranosyl halides was obtained by studying their reactions with methanol in the presence and absence of acid-acceptors. As expected the triacyl-β-D-ribopyranosyl halides reacted with the alcohol in the presence of acid-acceptors to yield acid-labile dextrorotatory syrups which most probably contained ortho-ester derivatives, whereas the α-halides gave acylated derivatives of methyl β-D-ribopyranoside in good yield. The latter result is in conformity with present ideas on the role of neighboring groups in replacement reactions (see Remick[147]) since the α-halides, having a halogen on C-1 in a *cis*-position relative to the benzoyloxy group on C-2 might be expected to react with methanol with simple inversion to give methyl β-D-riboside tri-*O*-benzoate. The β-halides, on the other hand, having a *trans*-relationship between the groups at C-1 and C-2, react with methanol, in part at least, by a different mechanism. (Cf. Jeanloz and Fletcher[1] for further details.) The *trans*-halide reacted more rapidly with methanol than its *cis*-isomer as shown in Table III.[146] It was shown[146] that tri-*O*-benzoyl-β-D-ribopyranosyl bromide in dry benzene-ether solution could be converted in high yield to the corresponding chloride by treatment with active silver chloride[148, 149] according to the method developed originally by Haworth *et al.*[150]

Levene and Tipson[110] found that tri-*O*-acetyl-β-D-ribopyranosyl bromide reacts with methanol in the presence of silver carbonate to give 3,4-di-*O*-acetyl-D-ribopyranose methyl 1,2-orthoacetate. Klingensmith and Evans[102] obtained an analogous compound (i.e., di-*O*-acetyl-D-ribose 1,2-ortho-3′-acetoxyacetonyl acetate) on condensing the same halide with dihydroxyacetone monoacetate. The structures of these compounds have recently been discussed by Pacsu.[151]

c. *N-Glycosides*

N-Ribosides occur naturally in important biological substances, as for example ribonucleic acids, vitamin B_{12} and cozymase. Furthermore in the ribityl derivatives which are found in Nature, C-1 of the reduced sugar residue is linked to a nitrogen atom in a heterocyclic nucleus. Consequently it is not surprising that a great deal of current interest in the chemistry of D-ribose is centered on the *N*-ribosides. The product from the condensation of D-ribose (XXIV) with ammonia or a primary amine might be expected

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[147] A. E. Remick, "Electronic Interpretations of Organic Chemistry," 2nd ed., p. 339. J. Wiley & Sons, New York, 1949.

[148] H. H. Schlubach, *Ber.* **59,** 840 (1926).

[149] H. H. Schlubach and R. Gilbert, *Ber.* **63,** 2292 (1930).

[150] W. N. Haworth, E. L. Hirst, and M. Stacey, *J. Chem. Soc.* **1931,** 2864.

[151] E. Pacsu, *Advances in Carbohydrate Chem.* **1,** 77 (1945).

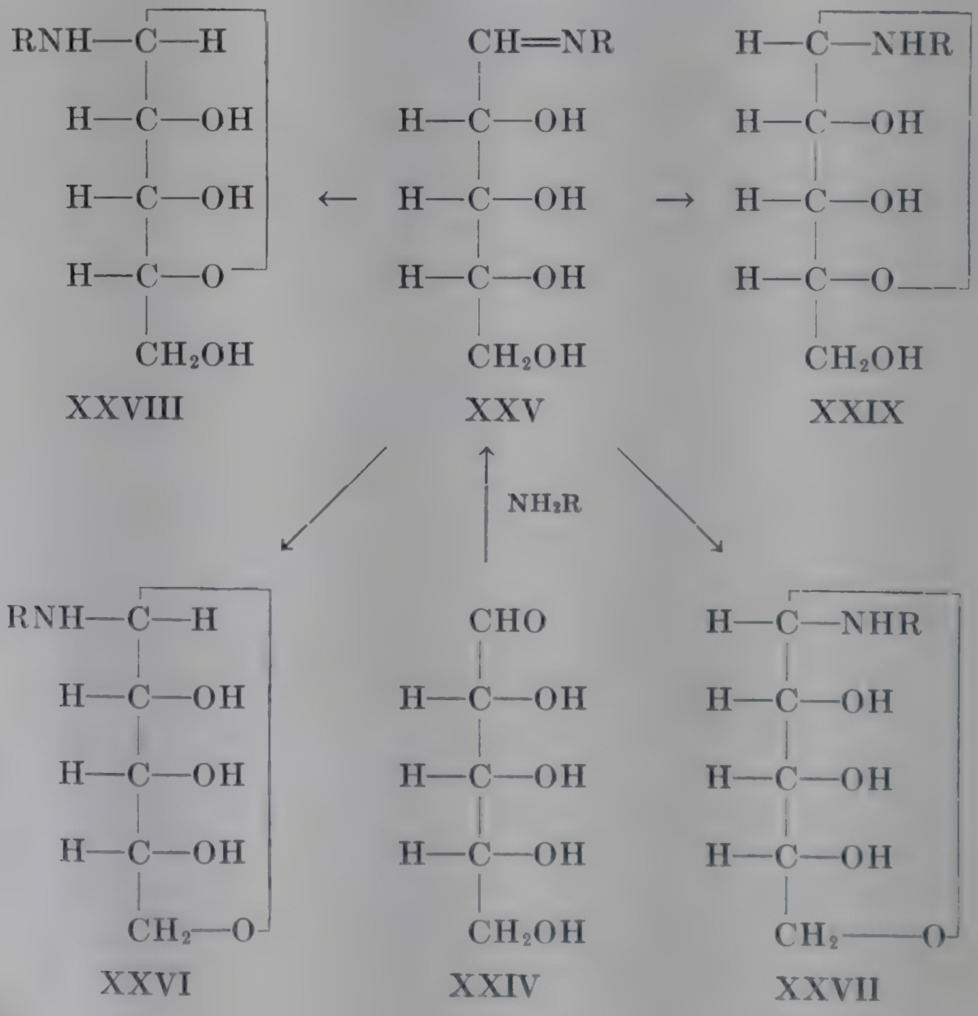
TABLE III

Reaction of Tri-O-benzoyl-D-ribopyranosyl Halides with Dioxane-Methanol (1:9) at 20°

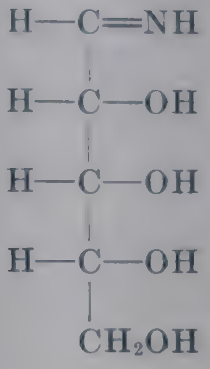
Compound	$K \times 10^4$ min., $\log_{10}$	Time of "half-change," min.	Final $[\alpha]_D^{20}$[a]
Tri-*O*-benzoyl-α-D-ribopyranosyl bromide	40	75	−68°
Tri-*O*-benzoyl-β-D-ribopyranosyl bromide	760	4.0	−53°
Tri-*O*-benzoyl-α-D-ribopyranosyl chloride	0.62	4900	−65°
Tri-*O*-benzoyl-β-D-ribopyranosyl chloride	53	57	−49°

[a] Calculated on the assumption that each halide was quantitatively converted to methyl pentoside tri-*O*-benzoate. Methyl β-D-ribopyranoside 2,3,4-tri-*O*-benzoate shows $[\alpha]_D^{20}$—65.2° in dioxane-methanol (1:9).

to exist in a variety of tautomeric structures, since the Schiff base (*syn*- or *anti*-form of XXV) contains potentialities for isomerism and might conceivably tautomerize to the anomeric pyranosides (XXVI and XXVII) or the anomeric pair of furanosides (XXVIII and XXIX). These possible changes have led to some confusion in explanations of the behavior of *N*-ribosides.



The simplest type of compound of this class, namely that formed by reaction between ammonia and D-ribose, was investigated by Levene and La Forge.[152] D-Ribose was treated with dry methanolic ammonia and the ribosimine (XXX or one of its cyclic tautomers) obtained was further treated with hydrogen cyanide in an attempted Kiliani-type synthesis of two epimeric hexosaminic acids. When less rigorously anhydrous conditions were employed, a diribosylamine was obtained, but neither of these derivatives of D-ribose has been thoroughly studied. Interest in the synthesis of vitamin B_2 led to the investigation of arylamine-N-ribosides. A brief but adequate review of the products of reaction between D-ribose and aniline was provided recently by Honeyman and Tatchell[153] In 1937 Kuhn and Ströbele[154] condensed D-ribose with 2-nitro-4,5-dimethylaniline by heating in alcoholic solution in the presence of 2–3 % ammonium chloride. The product on acetylation yielded a triacetate and gave a monotrityl derivative in high yield on tritylation and on the basis of these results was considered to be *N*-2-nitro-4,5-dimethylphenyl-D-ribofuranosylamine. Owing to the possibility of isomerizations taking place, during both the acetylation and tritylation reactions, the arguments in favor of the furanose structure cannot be considered convincing. Moreover, it is well known that trityl chloride will react with secondary hydroxyl groups in certain circumstances[155] and is not a specific reagent for primary hydroxyl groups.



$$\begin{array}{c}
\mathrm{H{-}C{=}NH} \\
| \\
\mathrm{H{-}C{-}OH} \\
| \\
\mathrm{H{-}C{-}OH} \\
| \\
\mathrm{H{-}C{-}OH} \\
| \\
\mathrm{CH_2OH}
\end{array}$$

XXX

An extensive series of investigations concerning the reactions of arylamines, and in particular aniline, with D-ribose was reported in 1945–46 by Berger, Lee and coworkers. They concluded that condensation at low temperatures of arylamines with D-ribose in alcoholic or aqueous alcoholic solution, with traces of acid as catalyst, yields *N*-aryl-α-D-ribopyranosylamine. If the reactants are condensed at the reflux temperature of the solvent *N*-aryl-α-D-ribofuranosylamines are formed. Configurational assignments were based on mutarotation studies on the products. In view of subsequent work by Barclay *et al.*,[156] this is a doubtful criterion for distinguishing between α- and β-anomers of *N*-glycosides. The *N*-aryl-D-ribopyranosylamines could be converted quantitatively to the corresponding *N*-aryl-D-ribofuranosylamines by heating in boiling alcoholic solution. Hydrogenation of both the pyranose and furanose forms of the arylamine-*N*-ribosides afforded the ribitylamine derivative.[89, 157, 158] Working with isomers of *N*-phenyl-D-ribosylamine, Howard *et al.*[159] found that the optical rotation of both isomers (i.e., pyranose and furanose isomers) is constant in dry pyridine and that a trace of moisture is necessary for mutarotation. Under these latter circumstances the two isomers do not come to the same end-value for the specific rotation and it was considered likely that each is undergoing α,β-isomerism. On the other hand, in solution in pyridine containing 10% acetic acid, the isomers are claimed to mutarotate to the same end-point. It was thought that an equilibrium involving change in ring size is established under these conditions and acylation experiments supported this viewpoint. Acetylation of both *N*-phenylribosylamines led to the same tri-*O*-acetyl-*N*-phenyl-D-ribosylamine which upon hydrolysis with methanolic ammonia afforded *N*-phenyl-D-ribopyranosylamine. Evidently the ribofuranosylamine had rearranged to the ribopyranosylamine during acetylation. Acidic hydrolysis of tri-*O*-acetyl-*N*-phenyl-D-ribopyranosylamine removed the aniline residue, and upon further acetylation of the sugar moiety β-D-ribopyranose tetra-*O*-acetate was obtained. The mechanism of isomerization of amine glycosides was discussed by Howard *et al.*[159] Measurements of the changes in the optical rotation of solutions of *N*-phenyl-D-ribosylamines have also been reported by Stacey and his colleagues.[160] In addition these workers also measured rates of acidic hydrolysis of these compounds. Recent experiments by Barclay *et al.*[156] suggest that care must be exercised in attributing to true mutarotational phenomena the changes in optical rotation that may occur in solutions of *N*-glycosides without prior ascertainment of the effect of water and pH alterations, since frequently the changes in rotation are caused by hydrolysis rather than mutarotation.

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[152] P. A. Levene and F. B. La Forge, *J. Biol. Chem.* **20**, 433 (1915).

[153] J. Honeyman and A. R. Tatchell, *J. Chem. Soc.* **1950**, 967.

[154] R. Kuhn and R. Ströbele, *Ber.* **70**, 773 (1937).

[155] R. C. Hockett and C. S. Hudson, *J. Am. Chem. Soc.* **56**, 945 (1934).

[156] J. L. Barclay, A. B. Foster, and W. G. Overend, *Chemistry & Industry* **1953**, 462.

Very recently the reaction between D-ribose and aniline has been reexamined critically by Ellis and Honeyman.[161] It was established that water has an influence in determining which isomer is obtained. Contrary to the suggestions of previous workers, temperature is not important in influencing the nature of the product. For example, the isomer (A) thought to be *N*-phenyl-D-ribopyranosylamine was produced either at room temperature or at the boiling point when aqueous ethanol was used as solvent. The presence of moisture in the condensation mixture always resulted in the production of this isomer and the supposed *N*-phenyl-D-ribofuranosylamine (B) was obtained only in anhydrous ethanol. Similarly, even a small amount of water prevents the conversion of isomer (A) into isomer (B) in boiling ethanol, and this probably explains some of the inconsistencies reported by Howard *et al.*[159] Contrary to workers in other laboratories, Ellis and Tatchell found no mutarotation for these isomers when in pyridine solution, even on addition of a drop of water. It will be recalled that it was largely on the basis of the mutarotation that Berger and Lee considered the isomers to have different lactol rings.

A similar cycle of reactions was carried out with D-ribose and *p*-toluidine.[161] Until the experiments of Ellis and Honeyman, the only known *N*-*p*-tolyl-D-ribosylamine was that prepared and described by Berger and Lee.[87] It was obtained by reaction at room temperature, in a solvent of ethanol containing a trace of water. Ellis and Honeyman obtained two isomers according to whether anhydrous or moist conditions

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[157] L. Berger and J. Lee, *J. Org. Chem.* **11,** 75 (1946).

[158] J. Lee, U. V. Solmssen, and L. Berger, U. S. Pat. 2,384,102 (Sept. 4, 1945).

[159] G. A. Howard, G. W. Kenner, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1946,** 855.

[160] K. Butler, S. G. Laland, W. G. Overend, and M. Stacey, *J. Chem. Soc.* **1950,** 1433.

[161] G. P. Ellis and J. Honeyman, *Nature* **167,** 239 (1951); *J. Chem. Soc.* **1952,** 1490.

of preparation were employed. The conditions of interconversion of these two isomers followed exactly those found to be satisfactory for interconverting the *N*-phenyl-D-ribosylamines. Likewise two isomers of *N*-*o*-nitrophenyl-D-ribosylamine were synthesized. The absorption of ultraviolet light by both isomers of *N*-*p*-tolyl-D-ribosylamine in methanolic solution was measured during the time mutarotation changes were occurring. There was no appreciable change in the absorption corresponding to the change in optical rotation which occurred. The absorption curve closely resembled those obtained with simple aromatic amines, the main difference being a slight lateral shift of the maxima [e.g., *N*-*p*-tolylribosylamine (formed in the absence of water) has $\lambda_{max.}$ 2450 (log ϵ 4.47) and 2900 A. (log ϵ 3.72) whereas the other isomer shows $\lambda_{max.}$ 2500 (log ϵ 5.62) and 2940 A. (log ϵ 4.25). Aniline is reported[162] to have $\lambda_{max.}$ 2300 (log ϵ 3.90) and 2800 A. (log ϵ 2.30)]. Details of other arylamine-*N*-ribosides are listed in the Appendix (Table VII). Reference has already been made to the ability of arylamine-*N*-ribopyranosides to form "complex salts" with the soluble salts of alkali metals.

Ribobenzimidazole [2-(D-*ribo*-1,2,3,4-tetrahydroxybutyl)benzimidazole] has been used for the characterization of this pentose and as a means of identifying the sugar component of yeast nucleic acid.[19-21] These experiments were described in Section III. (2). Recently interest in ribosylbenzimidazole derivatives has been stimulated by the finding that 5,6-dimethylbenzimidazole-α-D-ribofuranoside (XXXI) is a component part of the molecule of vitamin B_{12}. Folkers and his colleagues[34, 35] and workers in other laboratories[36-38] succeeded in degrading vitamin B_{12} by acidic hydryolsis to XXXI, the structure of which was confirmed by synthesis.[34] 2-Nitro-4,5-dimethylaniline (XXXII) was condensed with 5-O-trityl-D-ribofuranose (XXXIII) to give 2-nitro-4,5-dimethyl-*N*-(5′-trityl-D-ribofuranosido)aniline (XXXIV) or one of its tautomers. Successive hydrogenation and condensation of XXXIV with ethyl formimino ether hydrochloride and acid hydrolysis yielded 5,6-dimethylbenzimidazole-α-D-ribofuranoside (XXXI), isolated as the crystalline picrate. When XXXIV was acetylated prior to conversion to the benzimidazole derivative, the final product obtained after the above sequence of reactions and deacetylation was an isomer of XXXI. Optical rotation data suggested that it was the β-isomer. For both isomers the furanose structure for the sugar moiety was confirmed by periodate titration. For convenience the dextrorotatory isomer (XXXI) was termed α-ribazole and the levorotatory isomer was referred to as β-ribazole. Natural and synthetic α-ribazole had rat animal protein factor activity of about $\frac{1}{400}$ of that displayed by vitamin B_{12}.[163] At the level used β-ribazole had about the same activity as the α-isomer. Wacker and Weygand[164] have studied β-ribazole as an inhibitor of *Lactobacillus leichmannii* 313.

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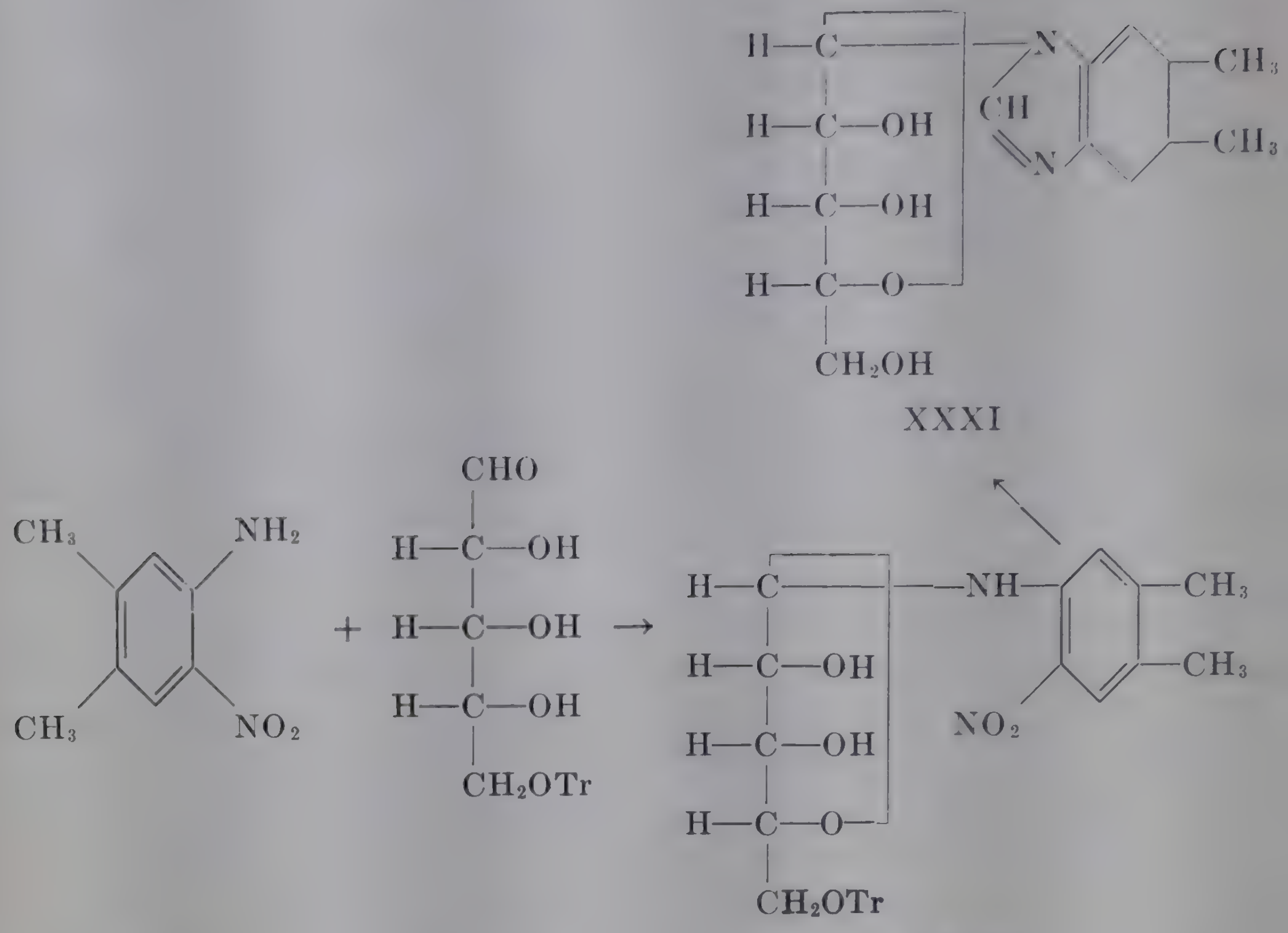
[162] For references see *Ann. Rpts. on Progr. Chem.* (*Chem. Soc. London*) **42,** 124 (1945).

[163] Gladys Emerson, F. W. Holly, C. H. Shunk, N. G. Brink, and K. Folkers, *J. Am. Chem. Soc.* **73,** 1069 (1951).

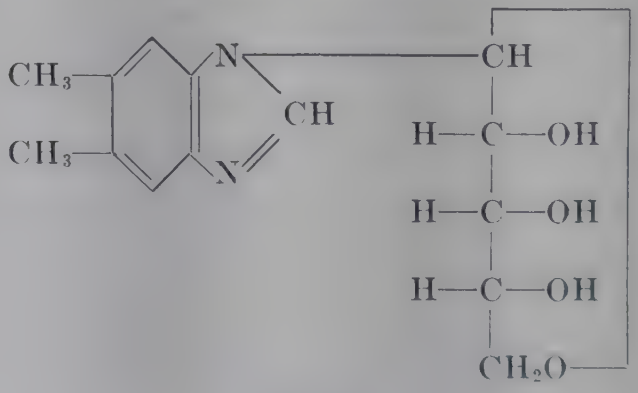
[164] A. Wacker and F. Weygand, *Z. Naturforsch.* **6b,** 130 (1951).



XXXI

XXXII XXXIII XXXIV

Subsequently Folkers and co-workers[165] prepared 5,6-dimethylbenzimidazole-α- and -β-D-ribopyranoside. The β-pyranoside (XXXV) has also been synthesized by Petrow *et al.*[166] The pyranose structure was confirmed by periodate titration and the β-configuration of the sugar-base linkage was assumed from the method of synthesis, i.e., that 5,6-dimethylbenzimidazole silver and α-acetobromoribose react with concomitant Walden inversion to give the acetate of XXXV, which would thus have a β-linkage.



XXXV

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[165] F. W. Holly, C. H. Shunk, Elizabeth W. Peel, J. J. Cahill, J. B. Lavigne, and K. Folkers, *J. Am. Chem. Soc.* **74,** 4521 (1952).

[166] G. Cooley, B. Ellis, P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm. and Pharmacol.* **2,** 579 (1950).

Petrow and his colleagues[167] measured the pK_a at 25 ± 1° of 5,6-dimethylbenzimidazole-1-β-D-ribopyranoside and found a value in water of 4.70 at a dilution of 490 liters per mole. Benzimidazole glycosides are less basic than the corresponding free benzimidazoles and in general the basicity falls in the series

1-β-D-ribo- > 1-α-D-arabo- > 1-α-L-arabo- > 1-β-D-xylo-pyranose.

Various methods have been used in connection with syntheses of riboflavin-type compounds to prepare *N*-ribityl derivatives.[168, 169] *N*-3,4-Dimethylphenyl-D-ribosylamine affords directly on hydrogenation 3,4-dimethylphenyl-D-ribamine.[170] Ribitylbenzimidazole derivatives have been prepared as possible carcinolytic compounds: 1-(1′-D-ribityl)-5,6-dimethylbenzimidazole was ineffective in producing regression of established lymphosarcoma implants in mice.[171] Because of the great interest in the naturally occurring nucleosides, studies of the synthesis of D-ribosides of purine and pyrimidines have been numerous [cf. *Baddiley*, Chapter 4.].

d. Phosphates

Many biochemical reactions proceed by the agency of phosphorylated derivatives of D-ribose (for reviews see Avison and Hawkins,[172] and Foster *et al.*[173]). [Cf. *Glock*, Chapter 22, and *Schlenk*, Chapter 24.] The present account will be limited mainly to a description of the chemistry of the monoorthophosphoric esters of ribose. Brief reference will be made to other pentose phosphates, both for comparative purposes and because some were suggested as components of nucleic acid, prior to the recognition of the sugar moiety as D-ribose. For comparison of the properties and reactions of pentose phosphates with those of hexose phosphates, disaccharide phosphates, etc., reference should be made to the excellent review of sugar phosphates by Leloir.[2]

Several methods are available for differentiating between the various phosphate esters of ribose. Like other aldose-1-phosphates, ribose-1-phosphate is very sensitive to acid treatment[174] and in this respect resembles the *O*-glycosides. Furthermore, like the glycosides, the β-anomers of aldose-1-

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[167] M. T. Davies, P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm. and Pharmacol.* **3,** 420 (1951).

[168] M. Tishler and J. W. Wellman, U. S. Pat. 2,261,608 (Nov. 4, 1941).

[169] R. Pasternack and E. V. Brown, U. S. Pat. 2,237,263 (April 1, 1941).

[170] R. Kuhn and L. Birkofer, *Ber.* **71,** 621 (1938).

[171] F. W. Holly, Elizabeth W. Peel, J. J. Cahill, and K. Folkers, *J. Am. Chem. Soc.* **73,** 332 (1951).

[172] A. W. D. Avison and J. D. Hawkins, *Quart. Revs.* (*London*) **5,** 171 (1951).

[173] A. B. Foster, W. G. Overend, and M. Stacey, *Die Stärke* **5,** 285 (1953).

[174] H. M. Kalckar, *J. Biol. Chem.* **167,** 477 (1947).

TABLE IV

Rates of Acidic Hydrolysis of Some Pentose Phosphates

Compound	Normality of acid	Temp., °C.	$K \times 10^3$[a]	Reference
Ribose-1-phosphate	0.50	25	1200	174
Ribose-3-phosphate	0.01	100	~1.7	175
	0.25	100	~4.5	176
Ribose-5-phosphate	0.01	100	~0.3	175
	0.25	100	~0.5	176
Xylose-1-phosphate	0.10	36	6.21	177
Xylose-5-phosphate	1.00	100	3–4	178

[a] Constants are calculated from the formula $K = \frac{1}{t} \log_{10} \frac{a}{a - x}$ or more usually $K = \frac{1}{t_2 - t_1} \log_{10} \frac{a - x_1}{a - x_2}$, when a is the initial concentration of the substance and the time (t) is expressed in minutes.

TABLE V

Specific Rotations of some Pentose Phosphate Esters

Compound	Salt	Solvent	$[\alpha]_D$	Reference
D-Ribose-3-phosphate	disodium	water	−9.7°	179
	disodium	0.5 saturated boric acid	+38°	179
D-Ribose-5-phosphate	free acid	water	+16.5°	175
	barium	water	+5°	180
D-Xylose-1-phosphate	barium	water	+65°	177
	dipotassium	water	+76°	177
D-Xylose-5-phosphate	disodium	water	+3.2°	178
	disodium	0.5 saturated borax	+4°	178
D-Arabinose-5-phosphate	barium	water	−18.8°	181
	brucine	50% aq. pyridine	−48.6°	181

phosphates are usually more acid-labile than the α-forms. Levene and Stiller[175] demonstrated that a pentose-3-phosphate hydrolyzes much more rapidly in acid solution than does the 5-isomer. For example, ribose-3-phosphate hydrolyzes 5 to 9 times faster than ribose-5-phosphate under identical conditions. This method of distinguishing between the 3- and 5-phosphate esters of ribose is applicable not only to the compounds themselves, but also to substances containing either of them as a molecular component.[45] Table IV includes values calculated for the velocity constant ($K \times 10^3$) for the acidic hydrolysis of ribose phosphates, and for comparison some xylose phosphates are included.

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[175] P. A. Levene and E. T. Stiller, *J. Biol. Chem.* **104,** 299 (1934).

[176] H. G. Albaum and W. W. Umbreit, *J. Biol. Chem.* **167,** 369 (1947).

[177] W. R. Meagher and W. Z. Hassid, *J. Am. Chem. Soc.* **68,** 2135 (1946).

[178] P. A. Levene and A. L. Raymond, *J. Biol. Chem.* **102,** 347 (1933).

[179] P. A. Levene and S. A. Harris, *J. Biol. Chem.* **95,** 755 (1932).

[180] A. M. Michelson and A. R. Todd, *J. Chem. Soc.* **1949,** 2476.

[181] P. A. Levene and C. C. Christman, *J. Biol. Chem.* **123,** 607 (1938).

Details concerning the alkaline hydrolysis of pentose phosphates are somewhat more scanty. Measurements of specific rotations have been used for distinguishing between ribose-3- and -5-phosphates and other pentose phosphates. In Table V values of the specific rotation for various pentose phosphates are listed.

Klimek and Parnas[182] distinguished between adenylic acids possessing ribose-3- and -5-phosphate moieties by a method based on the formation of a blue soluble complex by adenosine-5′-phosphate in alkaline solution in the presence of copper sulfate. Under the same conditions only an insoluble precipitate is formed by adenosine-3′-phosphate, which after centrifuging leaves a clear colorless supernatant solution. The procedure was standardized more completely by Berlin and Westerberg.[183] Albaum and Umbreit[176] have developed a method for differentiating between ribose-3- and -5-phosphates and compounds containing them, by means of the orcinol pentose color reaction. The method is rapid and can be used on as low an amount as 10 μg. of phosphate ester. It cannot be used precisely on crude plant and bacterial extracts containing polysaccharides, since these alter the rate of color development. Paper chromatography provides a valuable micromethod for the identification of sugar-phosphate esters.[184-187] Some R_F values of ribose phosphates and other pentose phosphates, as quoted by Cohen and McNair Scott,[185] are shown in Table VI.

The addition of boric acid to the solvents retards the movement of ribose-5-phosphate compared with arabinose-5-phosphate, an effect attributed to the combination of boric acid with the *cis*-hydroxyl groups attached to C-2 and C-3 in ribose. Ribose phosphates may be separated from other sugar phosphates by ion-exchange resin chromatography. [Cf. *Cohn*, Chapter 6.] Horecker and Smyrniotis[188] used Dowex 1 formate for the separation of pentose phosphates resulting from the action of a yeast enzyme on 6-phosphogluconate. Sugar phosphates have also been

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[182] R. Klimek and J. K. Parnas, *Biochem. Z.* **252,** 392 (1932).

[183] H. Berlin and J. Westerberg, *Z. physiol. Chem.* **281,** 98 (1944).

[184] C. S. Hanes and F. A. Isherwood, *Nature* **164,** 1107 (1949).

[185] S. S. Cohen and D. B. McNair Scott, *Science* **111,** 543 (1950).

[186] R. S. Bandurski and B. Axelrod, *J. Biol. Chem.* **193,** 405 (1951).

[187] A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka, *J. Am. Chem. Soc.* **72,** 1710 (1950).

[188] (a) B. L. Horecker and P. Z. Smyrniotis, *Arch. Biochem. and Biophys.* **29,** 232 (1950); see also (b) B. L. Horecker, P. Z. Smyrniotis, and J. E. Seegmiller, *J. Biol. Chem.* **193,** 383 (1951).

TABLE VI

R$_F$ VALUES OF PENTOSE PHOSPHATES

Compound	Solvent system	
	80% ethanol containing 0.8% acetate at pH 3.5	80% ethanol containing 0.64% boric acid
D-Ribose-5-phosphate	0.50	0
D-Arabinose-5-phosphate	0.54	0.25
D-Xylose-5-phosphate	0.55	0, 0.25
D-Ribose-3-phosphate	0.50	0, 0.19
D-Xylose-3-phosphate	0.53	0, 0.23

separated by ion exchange with the use of the borate complex,[189, 190] and it is also possible that paper ionophoresis can be used. [Compare Chapter 8.]

(1) *Ribose-1-phosphate.* Kalckar[174, 191-193] discovered that enzymic phosphorolysis of some nucleosides (inosine, guanosine) leads to the production of a pentose phosphate, isolable as its barium salt, which is most probably D-ribose-1-phosphate. [Cf. *Schlenk*, Chapter 24.] The yield of pentose phosphate generally was low (i.e., 7–10 mg. of the barium salt from 40–60 mg. of inosine). Probably losses are due to acid hydrolysis, to nonspecific and specific contaminant phosphatase action during the incubation with the enzyme and to retention on the bulky barium phosphate precipitate during the working up stage. Moreover the position of the equilibrium favors the formation of hypoxanthine riboside. Although Kalckar[194] was unable to demonstrate that adenosine or xanthosine or the pyrimidine ribosides would act in the system, it appears that the following relation might hold true:

$$\text{Ribose-1-purine} + PO_4^{---} \leftrightarrows \text{Ribose-1-phosphate} + \text{purine}$$

Subsequent work has demonstrated that in addition to the examples studied by Kalckar mammalian purine nucleoside phosphorylase catalyzes the synthesis from the respective base and ribose-1-phosphate of xanthosine,[195] 8-azaguanine riboside,[196] nicotinamide riboside[197] and 4-amino-5-imidazole-

[189] J. X. Khym and W. E. Cohn, *J. Am. Chem. Soc.* **75**, 1153 (1953).

[190] J. X. Khym, D. G. Doherty, E. Volkin, and W. E. Cohn, *J. Am. Chem. Soc.* **75**, 1262 (1953).

[191] H. M. Kalckar, *Federation Proc.* **4**, 248 (1945).

[192] H. M. Kalckar, *J. Biol. Chem.* **158**, 723 (1945).

[193] H. M. Kalckar, *Symposia Soc. Exptl. Biol.* **1**, 38 (1947).

[194] H. M. Kalckar, *Biochim. et Biophys. Acta* **4**, 232 (1950).

[195] M. Friedkin, *J. Am. Chem. Soc.* **74**, 112 (1952).

[196] M. Friedkin, *Federation Proc.* **11**, 216 (1952).

[197] J. W. Rowen and A. Kornberg, *J. Biol. Chem.* **193**, 497 (1951).

carboxamide riboside.[198] Moreover Korn *et al.*[198] working with purified beef liver nucleoside phosphorylase obtained some evidence of reaction between ribose-1-phosphate and adenine. This result differs from that obtained by Kalckar working with rat liver nucleoside phosphorylase.

An accumulation of evidence supports the designation of the pentose phosphate as ribose-1-phosphate. It is nonreducing and readily hydrolyzed by acid to equimolecular quantities of phosphate ions and pentose. The extreme acid lability supports a glycosidic phosphate ester linkage and indeed the substance is sufficiently acid-labile to lose its phosphate group by hydrolysis at the acidity employed in some of the methods available for phosphate estimation. The ester is somewhat more acid-labile than phosphocreatine but less so than acetyl phosphate. For example in 0.5 *N* sulfuric acid the half-time for splitting for phosphocreatine, acetyl phosphate and ribose-1-phosphate is 4 minutes, 30–40 seconds and 2.5 minutes, respectively. Further support for the glycosidic linkage is afforded by the enzymic conversion of the phosphate ester to purine ribosides which also have a linkage to C-1 of the sugar moiety. The stereochemical configuration of the phosphate ester linkage is unknown, but it is probably of the β-type. Natural nucleosides are considered to be β-furanosides,[144, 199] and, if nucleoside phosphorylase (like polysaccharide phosphorylase) produces no inversion,[200] the ribose-1-phosphate should also be of the β-furanose type. The furanose structure for the lactol ring is supported by the observation[194] that synthetic ribopyranose-1-phosphate is inactive as a substrate for nucleoside phosphorylase.

Ribose-1-phosphate can be converted enzymically by mutase action into ribose-5-phosphate. [Cf. *Glock*, Chapter 22.] Klenow and Larsen[201] have shown that phosphoglucomutase preparations,[202, 203] acting in conjunction with glucose-1,6-diphosphate (or possibly ribose-1,5-diphosphate) as coenzyme, will bring about this change. The ratio of the enzymic activities of phosphoglucomutase:phosphoribomutase was about 100:1. Reports by Wajzer and Baron[204] indicate that liver contains an enzyme capable of transforming ribose-1-phosphate into the -5-phosphate.

(2) *Ribose-2- and -3-phosphates.* The location of the phosphate residue in the first pair of isomeric nucleotides discovered and isolated by Cohn[99] (adenylic acids "a" and "b") has generally been regarded as at the 2′- and

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[198] E. D. Korn, F. C. Charalampous, and J. M. Buchanan, *J. Am. Chem. Soc.* **75,** 3610 (1953).

[199] J. Davoll, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1946,** 833.

[200] Mildred Cohn, *J. Biol. Chem.* **180,** 771 (1949).

[201] Hans Klenow and B. Larsen, *Arch. Biochem. and Biophys.* **37,** 488 (1952).

[202] V. A. Najjar, *J. Biol. Chem.* **175,** 281 (1948).

[203] E. W. Sutherland, *J. Biol. Chem.* **180,** 1279 (1949).

[204] J. Wajzer and Françoise Baron, *Bull. soc. chim. biol.* **31,** 750 (1949).

3′-positions, but not necessarily respectively.[205, 206] [Compare Chapters and 12.] The structures of the subsequently isolated isomeric pairs o guanylic,[99] cytidylic[207] and uridylic acids[207] have been assumed to be th same as the adenylic acid pair. The demonstrated acid-catalyzed migration of the phosphate group[205-207] made difficult a decision as to whicl nucleotide was 2′- and which 3′- in synthetic[208] and degradative[209] approaches to the problem. The problem has been solved in elegant fashior by Cohn *et al.*,[190] who succeeded in identifying the isomeric adenylic acid "a" and "b" as the adenosine-2′- and -3′-phosphates, respectively. They were able to hydrolyze catalytically the *N*-glycoside linkage of the individual adenylic acid isomers with the hydrogen form of a polystyrene sulfoni acid resin (Dowex 50) at a rate comparable to the rate of isomerization. The advantage of this method of hydrolysis lies in the fact that the ribose phosphates are released from the resin at the time of formation (in contrast t adenine and most of the adenylic acid) and, therefore, little or no isomerization takes place subsequent to their formation. The two ribose phosphates obtained were separated by an ion-exchange procedure.[189] The ribose phosphate "a" (derived from adenylic acid "a") could be converted to a methyl phosphoribopyranoside which consumed one mole of periodate, and to a ribitol phosphate with a marked optical activity which is enhanced by borate. The reverse properties (i.e., no periodate oxidation of the methyl phosphoriboside, no optical activity of the ribitol phosphate with or without borate) were noted for the "b" ribose phosphate. The possibility of the 1- or 5-phosphate isomers arising was excluded by the ion-exchange behavior of the substances, and the 4-phosphate ester is *a prior* excluded by the furanoside structure of the parent nucleotide.[210] Hence it follows that ribose phosphate "a" and "b" are ribose-2- and -3-phosphate, respectively. This is the first isolation of ribose-2-phosphate. Moreove the work proved that Levene and his colleagues in their earlier structural studies of the purine nucleotides were dealing with the "b" isomers which would be expected to give rise to ribose-3-phosphate if no migration occurred during isolation.

Initial attempts by Levene and Jorpes[211] to prepare ribose-3-phosphat by acidic hydrolysis of adenosine-3′-phosphate, were unsuccessful a cleavage of the basic and phosphate residues proceeded at about the sam

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[205] D. M. Brown and A. R. Todd, *J. Chem. Soc.* **1952,** 44, 52.

[206] D. M. Brown, D. I. Magrath, and A. R. Todd, *J. Chem. Soc.* **1952,** 2708.

[207] W. E. Cohn, *J. Am. Chem. Soc.* **72,** 2811 (1950).

[208] D. M. Brown, L. J. Haynes, and A. R. Todd, *J. Chem. Soc.* **1950,** 408.

[209] D. G. Doherty, *Abstracts Papers 118th Meeting Am. Chem. Soc.* 56 (1950).

[210] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **94,** 809 (1932); **97,** 491 (1932); **101** 529 (1933).

[211] P. A. Levene and E. Jorpes, *J. Biol. Chem.* **81,** 575 (1929).

rate. However, Levene and his co-workers[179, 212] were able to isolate a crystalline dibrucine salt of a ribose phosphate (not identical with the known ribose-5-phosphate) from a hydrolysate of xanthylic acid. The xanthylic acid was obtained by treatment of guanylic acid with nitrous acid: after deamination the sugar-base linkage is found to be more labile. Reduction of the ribose phosphate yielded an optically inactive ribitol phosphate,[213] considered to be ribitol-3-phosphate, derived from ribose-3-phosphate. This structural proof makes no account for possible migration of the phosphate group during any of the experimental operations. Deamination of adenosine-3′-phosphate to the corresponding inosinic acid, followed by hydrolysis, also yields some ribose-3-phosphate.[214]

By methanolysis of yeast nucleic acid, Levene and Harris[214] obtained a crude sample of a methyl D-ribopyranoside-3-phosphate. Exhaustive methylation and subsequent dephosphorylation afforded methyl 2,4-di-*O*-methyl-D-riboside, which on successive hydrolysis and catalytic reduction yielded 2,4-di-*O*-methyl-D-ribitol. Although this compound is a *meso* structure and would be expected to be optically inactive, the product did exhibit some optical activity, and it was shown that the crude methyl D-ribopyranoside-3-phosphate was contaminated with a furanoside isomer which gave rise to optically active 2,5-di-*O*-methyl-D-ribitol as an impurity in the 2,4-di-*O*-methyl derivative.

LePage and Umbreit[215] have prepared ribose-3-phosphate by acidic hydrolysis of a pure adenosine triphosphate isolated from the autotrophic bacterium *Thiobacillus thio-oxydans*.

(3) *Ribose-5-phosphate*. Ribose-5-phosphate was first obtained by Levene and Jacobs[11] by subjecting the barium salt of inosinic acid to acidic hydrolysis. The pentose phosphate was isolated as the crystalline hydrated barium salt. Shortly afterwards the same workers[12] showed that oxidation of the pentose phosphate with either bromine or nitric acid yields a phosphoribonic acid. If position C-5 had been unsubstituted, nitric acid oxidation would have been expected to produce a trihydroxyglutaric acid, and so the phosphate residue was considered to be located at C-5 of the ribose molecule. Much later[216] further evidence was forwarded which substantiated this conclusion, since lactonization of the D-ribonic acid phosphate proceeded very slowly, equilibrium being reached only after 150 hours. This is the behavior expected of a pentonic acid substituted at C-5 and unable to form other than a $\gamma(1,4)$-lactone.[217, 218] Furthermore, reduction

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[212] P. A. Levene and A. Dmochowski, *J. Biol. Chem.* **93,** 563 (1931).

[213] P. A. Levene and S. A. Harris, *J. Biol. Chem.* **98,** 9 (1932).

[214] P. A. Levene and S. A. Harris, *J. Biol. Chem.* **101,** 419 (1933).

[215] G. A. LePage and W. W. Umbreit, *J. Biol. Chem.* **148,** 255 (1943).

[216] P. A. Levene and T. Mori, *J. Biol. Chem.* **81,** 215 (1929).

[217] P. A. Levene and H. S. Simms, *J. Biol. Chem.* **65,** 31 (1925).

of the pentose phosphate afforded an optically active phosphoribitol, a so position C-3 of the sugar molecule was excluded as the site of esterific tion. In solution in methanolic hydrogen chloride the pentose phospha underwent mutarotation in a manner characteristic of a sugar whi can only form a furanoside.[219] Moreover the behavior of the pentose pho phate isolated from natural sources and a synthetic sample of D-ribose- phosphate was identical. The synthetic material was prepared[175] from ribose by condensation with acetone and methanol in the presence hydrogen chloride and anhydrous copper sulfate (or alternatively dilu sulfuric acid) followed by phosphorylation of the resultant syrupy meth 2,3,-*O*-isopropylidene-D-ribofuranoside (XXXVI) with phosphorus ox chloride and pyridine at −40°. Hydrolysis of the isopropylidene a glycosidic groups from methyl 2,3-*O*-isopropylidene-D-ribofuranoside- phosphate (XXXVII) yielded ribose-5-phosphate (XXXVIII) (as bariu salt). The structure of the important intermediate XXVI was demo strated by methylation and hydrolysis to an amorphous monomethylribo (XXXIX) which was converted to a crystalline *p*-bromophenylosazon identical with the *p*-bromophenylosazone of authentic 5-*O*-methyl- ribose which had been previously prepared[220] (see p. 42).

Using as phosphorylating agent dibenzylphosphorochloridate in d pyridine solution at −40°, Michelson and Todd[180] considerably improv the synthesis. Protecting groups were removed from the intermediate X by hydrogenation and hydrolysis to give XXXVIII in 86% yield.

An improved method for the preparation from muscle (horse, dog a rabbit muscle are excellent sources) of inosinic acid and thence ribose- phosphate has recently been described.[221] Optimum conditions were dete mined for hydrolysis of the nucleotide to ribose phosphate which can obtained in a yield of 50–60% by use of this method. This phospha ester is also obtainable by acidic hydrolysis of cozymase.[45] Adenine a nicotinamide are cleaved quantitatively while 20% of the total phosphor is liberated. Adenine was removed as its silver salt and the ribose-5-pho phate was isolated as the barium salt. The isolation of this compou served to identify the sugar moiety in cozymase and also the site at whi it was esterified by the phosphoric acid residue. Similar conclusions we reached from less direct evidence by other investigators.[41] Ribose-5-pho phate has been obtained in a high degree of purity from adenosine t phosphate.[222] Purification was achieved by chromatography on ion-exchan

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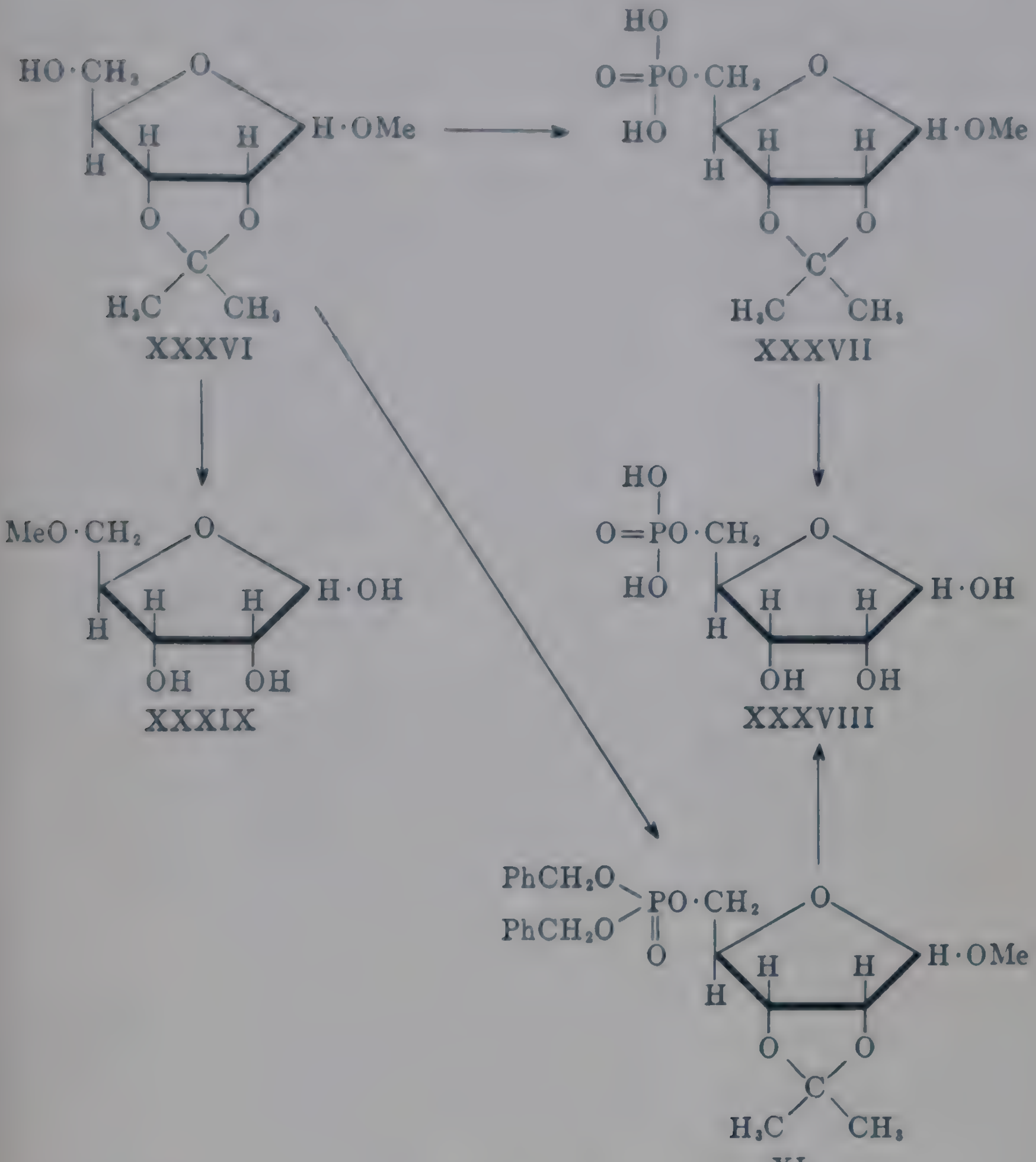
[218] P. A. Levene and M. L. Wolfrom, *J. Biol. Chem.* **77**, 671 (1928).

[219] P. A. Levene, S. A. Harris, and E. T. Stiller, *J. Biol. Chem.* **105**, 153 (1934).

[220] P. A. Levene and E. T. Stiller, *J. Biol. Chem.* **102**, 187 (1933).

[221] J. Marmur, F. Schlenk, and R. N. Overland, *Arch. Biochem. and Biophys.* **34**, (1951).

[222] D. P. Groth, G. C. Mueller, and G. A. LePage, *J. Biol. Chem.* **199**, 389 (1952).



esin (monochloroacetate of Dowex 1). The overall yield of the pentose hosphate from adenosine triphosphate was 63–70 %. As previously menioned, ribose-5-phosphate can be obtained from ribose-1-phosphate by the nutase action of liver[204] and yeast[201] extracts. D-Gluconic acid 6-phosphate nay be degraded enzymically to D-ribose-5-phosphate[185, 188] and this bservation is relevant to any discussion on possible routes[223] for the iogenesis of D-ribose. [Cf. *Glock*, Chapter 22.] Ribose-5-phosphate was ound to be vigorously metabolized by animal and yeast extracts,[224, 225] ut arabinose-5-phosphate and xylose-5-phosphate were only slightly ttacked.

(4) *Other Pentose Phosphates.* During the researches which led to the identification

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I. A. Bernstein, *J. Am. Chem. Soc.* **73**, 5003 (1951).

F. Dickens, *Biochem. J.* **32**, 1626, 1645 (1938).

F. Dickens and Gertrude E. Glock, *Nature* **166**, 33 (1950).

of D-ribose as the sugar component of yeast nucleic acid, several other pentose ph phates were prepared. Interest in xylose phosphate stemmed from Robinson's suggestion that xylose might be a primary constituent of nucleic acids and th ribose might result by hydrolysis of xylose-3-phosphate with Walden inversion. T possibility does not apply to ribose-5-phosphate isolated from inosinic acid becau a Walden inversion at the primary hydroxyl group would not affect the configurati of the sugar. Attempts by Levene and Raymond[178] to prepare xylose-3-phosphate f testing the validity of Robinson's hypothesis, were unsuccessful. Phosphorylati of xylose derivatives with only the hydroxyl group at C-3 free, gave xylose-5-ph phate derivatives, and obviously migration occurred at some stage of the preparati via an intermediate cyclic diester structure. The only 3-phosphate derivative whi they were able to isolate was 1,2-*O*-isopropylidine-3-phosphate-5-*O*-methylxylo Owing to the difficulty of removing the methyl group, this derivative was of lit value for comparison with the phosphopentose derived from nucleic acid. The ra of dephosphorylation for this compound was many times greater than that of xylos 5-phosphate.

Xylopyranose-1-phosphate (isolated as the barium or dipotassium salt) was pr pared by reacting bromoacetylxylose and trisilver phosphate and subsequent parti hydrolysis.[177]

Generally pentose phosphate esters are stronger acids than free phosphoric ac and the values of pK_1' and pK_2' are smaller. For example, the dissociation constan of xylose-1-phosphate calculated by means of Van Slyke's[227] formula and the He derson-Hasselbach equation are $pK_1' = 1.25$ and $pK_2' = 6.15$. Comparative valu for phosphoric acid are $pK_1' = 1.95\text{–}2.00$ and $pK_2' = 6.83\text{–}6.93$.[227-230]

D-Arabinose-5-phosphate was synthesized by Levene and Christman.[181]

(*e*) *Ethers, Esters, Acetals and Anhydrides.*

(1) *Ethers.* Reference has already been made to the complete methyl tion of methyl D-riboside and to the fact that acidic hydrolysis of t product yields crystalline 2,3,4-tri-*O*-methyl-D-ribose.[136, 231] Proof of stru ture followed from nitric acid oxidation which gave *i*-trimethoxyglutar acid. An isomeric tri-*O*-methyl-D-ribose was obtained by Levene a Tipson by subjecting either adenosine[210] or guanosine[210] to methylati and subsequent hydrolysis. The amorphous product reacted more rapid with acidic methanol than the 2,3,4-isomer and could be converted t γ-lactone. Nitric acid oxidation afforded *i*-dimethoxysuccinic acid, and the basis of these results the sugar was considered to be 2,3,5-tri-*O*-meth D-ribose, a conclusion subsequently confirmed by synthesis studies Methyl 2,3-*O*-isopropylidene-5-*O*-methyl-D-ribofuranoside was prepared successive acetonation and methylation of methyl D-ribofuranoside. H dolysis, further methylation and rehydrolysis furnished 2,3,5-tri-

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[226] R. A. Robinson, *Nature* **120,** 44 (1927).

[227] D. D. Van Slyke, *J. Biol. Chem.* **52,** 525 (1922).

[228] O. Meyerhof and J. Suranyi, *Biochem. Z.* **178,** 427 (1926).

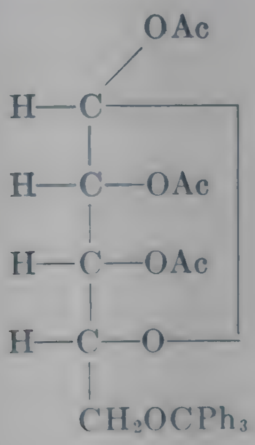
[229] C. F. Cori, S. P. Colowick, and Gerty T. Cori, *J. Biol. Chem.* **121,** 465 (1937).

[230] H. T. S. Britton and R. A. Robinson, *Trans. Faraday Soc.* **28,** 531 (1932).

[231] P. A. Levene and J. Compton, *J. Biol. Chem.* **116,** 169 (1936).

methyl-D-ribose, identical with that obtained from adenosine and guanosine. The structure of the synthetic material was rigidly established by the usual methods of carbohydrate chemistry. Further confirmation of the structure has since been provided by Barker.[134] Benzylation of methyl 2,3-*O*-isopropylidene-D-ribofuranoside afforded the 5-benzyl ether, which on hydrolysis was converted to syrupy 5-*O*-benzyl-D-ribose.[232] Originally it was reported by Bredereck *et al.*[96] that direct tritylation of D-ribose in pyridine solution yields crystalline 5-*O*-trityl-α-D-ribose, and Barker and Lock[233] demonstrated that acetylation without prior isolation of the trityl derivative tripled the yield of acetylated trityl ether. An improved alternative preparation of 5-*O*-trityl-α-D-ribofuranose was reported by Zinner.[113] In a subsequent publication Bredereck and Greiner[234] described methods for the preparation of several trityl ethers (e.g., 1-, 5-, 1,3(2?)-, 1,5- and 1,3,5-) of D-ribose and of their acetyl and benzoyl derivatives.

(2) *Esters.* The acetates and benzoates of D-ribose have been extensively investigated. Acetylation in pyridine solution at low or ordinary temperatures results in the formation of crystalline 1,2,3,4-tetra-*O*-acetyl-β-D-ribopyranose.[33, 110, 235] When acetic anhydride and sodium acetate were used as acetylating agents at higher temperature the product was 1,2,3,5-tetra-*O*-acetyl-D-ribofuranose.[141] Zinner[141] investigated the acetylation with acetic anhydride of ribose in pyridine solution at various temperatures and found that increase in the reaction temperature is accompanied by more formation of the furanose isomer, so that at 100° the proportions of the furanose and pyranose forms are approximately equal. The first synthesis of crystalline D-ribofuranose tetraacetate was accomplished successfully by Howard *et al.*[144] by reductive detritylation of 1,2,3-tri-*O*-acetyl-5-*O*-trityl-D-ribo furanose (XLI) and subsequent acetylation. The tetraacetate is



XLI

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[232] G. W. Kenner, C. W. Taylor, and A. R. Todd, *J. Chem. Soc.* **1949,** 1620.

[233] G. R. Barker and M. V. Lock, *J. Chem. Soc.* **1950,** 23.

[234] H. Bredereck and W. Greiner, *Chem. Ber.* **86,** 717 (1953).

[235] H. Zinner, *Chem. Ber.* **86,** 817 (1953).

a useful intermediate in the synthesis of naturally occurring ribonucleosides and this offers some confirmation for the structure assigned. The method was improved, so that the product could be isolated directly by accomplishing simultaneous detritylation and acetylation of XLI with acetyl bromide in acetic anhydride.[236] 5-*O*-Benzyl-D-ribofuranose can also be used as an intermediate for the preparation of this acetate.[232]

Some confusion has arisen in the literature concerning D-ribofuranose tetra-*O*-acetate. Thus, for this compound Howard *et al.*[144] report m.p. 58° and $[\alpha]_D^{15}$ +20° (in chloroform), Bredereck and Hoepfner[236] state that the physical constants are m.p. 56° and $[\alpha]_D^{20}$ −3.6° (in methanol), whereas Zinner[141] describes a product of m.p. 82° and $[\alpha]_D$ −12.6° (in chloroform) and −15.4° (in methanol). Zinner[141, 235] referred to his product as β-tetra-*O*-acetylribofuranose, and it obviously differed from the acetate prepared by Howard *et al.*[144] and Bredereck and Hoepfner.[236] Davoll *et al.*[237] have described some experiences with the preparation of this acetate by the Bredereck method. The first three portions prepared had m.p. 56–58°, but the fourth and all subsequent batches had m.p. 85° and $[\alpha]_D^{23}$ −12° (in chloroform) and −13.5° (in methanol), and were obviously identical with the product prepared by the method of Zinner. After the isolation of the latter product the melting points of earlier samples spontaneously changed to 85° and it became impossible to prepare samples of the acetate of lower melting point. Similarly, samples of products of lower m.p. forwarded to Davoll from other laboratories changed into the higher melting form. According to Davoll *et al.*[237] the change was accompanied by a change in optical rotation. The furanose structure of the material of higher melting point was proved by its conversion through the aceto-chloro-compound to adenosine in yields comparable to those obtained using tetra-*O*-acetyl-D-ribofuranose of m.p. 56–58° as the initial material. Davoll and his colleagues were unable to determine the difference between the isomers, but did not think that it was due to simple α,β-isomerism, since according to them measurements of optical rotation changes indicated that a complex process was operating in which at least three molecular species were involved. They considered it possible that one of the isomers had an orthoacetate anhydride structure, but this is difficult to reconcile with the stability of both forms to water and ethanol. Farrar[237a] considers that speculations regarding possible structures of the two forms are unnecessary, and all that is involved is a simple, but interesting, case of dimorphism, since she found that isomerization proceeded without any significant change in optical rotation. Obviously a contradiction exists between this result and the rotation changes reported by Davoll *et al.*,[237] and the problem needs to be investigated further.

2,3,4,5-Tetra-*O*-acetyl-*aldehydo*-D-ribose has been obtained crystalline and can be prepared by several routes. Demercaptalation of 2,3,4,5-tetra-*O*-acetyl-D-ribose diethyl thioacetal,[238] hydrogenolysis with Raney nickel of ethylthio-D-ribonate tetra-*O*-acetate[239] or Rosenmund reduction of tetra-*O*-acetyl-D-ribonyl chloride, all yield this compound.[169] Attempts to

[236] H. Bredereck and Eva Hoepfner, *Chem. Ber.* **81,** 51 (1948).

[237] J. Davoll, G. B. Brown, and D. W. Visser, *Nature* **170,** 64 (1952).

[237a] Kathleen R. Farrar, *Nature* **170,** 896 (1952).

[238] H. Zinner, *Chem. Ber.* **83,** 418 (1950).

[239] M. L. Wolfrom and J. V. Karabinos, *J. Am. Chem. Soc.* **68,** 724, 1455 (1946).

prepare 2,3,5-tri-*O*-acetyl-D-ribose by hydrolysis of arylamine derivatives have afforded only crude amorphous samples of the ester.[88, 90, 169] Recently, Zinner[235] has reported the preparation of all the tetra-*O*-acetates of D-ribose.

Methods for obtaining tetra-*O*-acetyl-D-ribonic acid have been investigated by Tishler *et al.*[240] Acetylation of the acid according to the procedure of Robbins and Upson[241] resulted in the production of some tetra-*O*-acetyl-D-ribonic acid in low yield (15%), admixed with tri-*O*-acetyl-D-ribonolactone (10%) and an intractable oil. A method was developed which gave the required acetate in high yield. Cadmium *D*-ribonate was treated at 10° with acetic anhydride and hydrogen chloride and the crystalline ribonic acid tetra-*O*-acetate was obtained in 85% of the theoretical yield. The cation is important in this acetylation, as shown by the varying yields of product obtained when other salts were used. When made to react with phosphorus oxychloride in chloroform, tetra-*O*-acetyl-D-ribonamide was converted to tetra-*O*-acetyl-D-ribononitrile.[240]

Cation..................	Ba^{++}	Ca^{++}	K^+	NH_4^+	Cd^{++}
Yield of product (%)....	4	22	25	46	85

Knowledge of the benzoates of D-ribose is due mainly to the researches of Hudson, Fletcher and their co-workers. A crystalline tetra-*O*-benzoate was obtained by benzoylation of the sugar in pyridine solution at low temperature.[111] This was considered to have the β-configuration and was demonstrated to have a pyranose structure by conversion into tri-*O*-benzoyl-D-ribosyl bromide, which was condensed with the potassium salt of 2-thionaphthol to give 2′-naphthyl-1-thio-β-D-riboside tri-*O*-benzoate. Desulfurization with Raney nickel and subsequent debenzoylation afforded an anhydroribitol in high yield. This product resembled 1,5-anhydro-D-xylitol[242] and 1,5-anhydro-D-arabitol[243] in its solubility characteristics. Neither the anhydro derivative nor its acetyl or benzoyl derivatives exhibited optical activity. This would be expected if it had a meso structure, as would be the case if the original tetra-*O*-benzoate had a pyranose(1,5)-lactol ring structure. Periodate oxidation on the anhydroribitol confirmed this lactol ring structure. Hydrolysis of 2,3,4-tri-*O*-benzoyl-D-ribosyl bromide with moist acetone in the presence of an acid-acceptor (Ag_2CO_3) yielded 2,3,4-tri-*O*-benzoyl-D-ribose.[145, 146] The isomeric 2,3,5-tri-*O*-benzoyl derivative has also been prepared in crystalline form.[244] Esters of

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[240] K. Ladenburg, M. Tishler, J. W. Wellman, and R. D. Babson, *J. Am. Chem. Soc.* **66**, 1217 (1944).

[241] G. B. Robbins and F. W. Upson, *J. Am. Chem. Soc.* **60**, 1788 (1938).

[242] H. G. Fletcher, Jr., and C. S. Hudson, *J. Am. Chem. Soc.* **69**, 921 (1947).

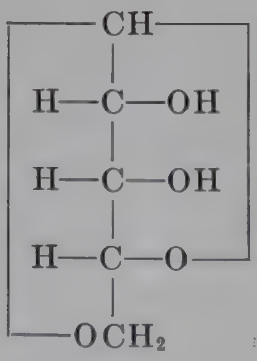
[243] H. G. Fletcher, Jr., and C. S. Hudson, *J. Am. Chem. Soc.* **69**, 1672 (1947).

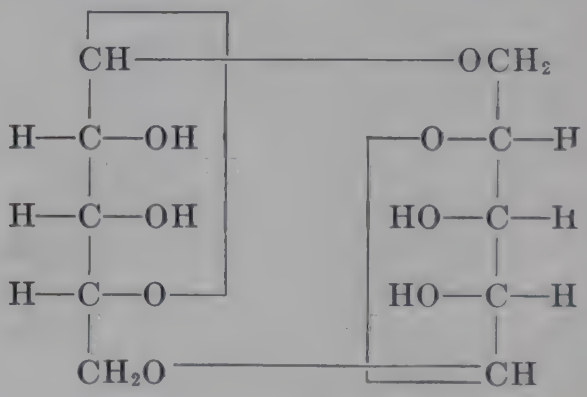
[244] R. K. Ness and H. G. Fletcher, Jr., *J. Am. Chem. Soc.* **75**, 3289 (1953).

D-ribose containing both acetate and benzoate groupings are known. Kenner *et al.*[245] synthesized 2,3,4-tri-*O*-acetyl-5-*O*-benzoyl-D-ribose, and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribose has been prepared from guanosine.[246]

(3) *Acetals.* Two products are obtained when D-ribose is treated with acetone in the presence of sulfuric acid and anhydrous copper sulfate,[220] one of which is crystalline and the other syrupy. The crystalline material is unreactive and is considered to be 2,3-*O*-isopropylidene-1,5-anhydro-D-ribofuranose. The syrupy material, which is the major product, can be purified via its di-*O*-acetyl derivative and by the usual methods of classical carbohydrate chemistry and has been shown to be 2,3-*O*-isopropylidene-D-ribofuranose. If D-ribose is treated with acetone and methanol in the presence of sulfuric acid and anhydrous copper sulfate, methyl 2,3-*O*-isopropylidene-D-ribofuranoside is obtained directly.[175] This compound has been used as an intermediate in syntheses of naturally occurring ribose derivatives. Attempts to acetonate methyl D-ribopyranoside gave mixtures of methyl mono-*O*-isopropylidene-D-ribo-furanoside and -pyranoside, and obviously some rearrangement of the lactol ring occurred during the reaction.[247]

(4) *Anhydrides.* In an attempt to prepare 1,2,3-tri-*O*-acetyl-D-ribofuranose by detritylation of 1,2,3-tri-*O*-acetyl-5-*O*-trityl-D-ribofuranose with hydrogen bromide in glacial acetic acid at 0°, Bredereck *et al.*[96] isolated an acetate which on hydrolysis afforded an anhydride of D-ribose. The structure assigned to this compound by these workers was D-ribosan⟨1,4⟩ β⟨1,5⟩ (XLII). The anhydride gave a positive test for vicinal hydroxyl groups and was reducing to Fehling's solution after acidic hydrolysis. Barker and Lock[233] and Jeanloz[248] reexamined the compound and found that it was bimolecular. A crystalline tetra-*O*-methyl-di-D-ribose


XLII


XLIII

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[245] G. W. Kenner, H. J. Rodda, and A. R. Todd, *J. Chem. Soc.* **1949**, 1613.
[246] F. Weygand and W. Sigmund, *Chem. Ber.* **86**, 160 (1953).
[247] P. A. Levene and E. T. Stiller, *J. Biol. Chem.* **106**, 421 (1934).
[248] R. W. Jeanloz, G. R. Barker, and M. V. Lock, *Nature* **167**, 42 (1951).

anhydride was prepared which hydrolyzed to 2,3-di-*O*-methyl-D-ribose. Oxidation of the anhydride with sodium metaperiodate was also investigated, and all the evidence was consistent with the structure of the compound being most probably 1,5′-5,1′-diribofuranose anhydride (XLIII).

As already briefly mentioned, acetonation of ribose in the presence of sulfuric acid and acetic anhydride leads to the production of a small amount of a product assumed to be 1,5-anhydro-2,3-*O*-isopropylidene-D-ribofuranose. If hydrogen chloride is used as condensing agent, an isomeric compound is obtained having a lower melting point.[101] Neither compound has been fully investigated. Methyl-2,3-anhydroribosides have been prepared by treatment of 2-*O*-tosyl or 2-*O*-mesyl derivatives of methyl arabinoside with sodium methoxide.[249-252] This anhydro compound has been used as an intermediate in attempted syntheses of 2-deoxyribose as described on pages 50–51. Hot aqueous sodium hydroxide solution converts methyl 2,3-anhydro-β-L-riboside into methyl β-L-xyloside (75%) and methyl β-L-arabinoside (25%).[249] The synthesis and establishment of structure of 1,5-anhydroribitol has already been described[111] (p. 45). When D-ribobenzimidazole is heated with zinc chloride and concentrated hydrochloric acid at 180°, anhydrization of the sugar molecule occurs to give 2-(1′,4′-anhydro-D-*ribo*-tetrahydroxybutyl)benzimidazole.[253]

(*f*) *Other Properties.*

D-Ribose reacts very readily with alkyl- and aryl-thiols in the presence of hydrochloric acid.[1, 114] Mention has already been made to the conversion of ribose into brom-acetyl (and benzoyl) ribose and to uses of the compounds. (See also Baxter *et al.*[254].) Various references to the action of oxidizing agents on this pentose have already been quoted during the course of this review. In general the reactions seem to proceed as expected. It has been reported[240] that D-ribonic acid is somewhat unstable at room temperature, as indicated by a lowering of the melting point by several degrees after storage for 24 hours. The acid can be converted into a lactone, the amide and esters. The tetra-*O*-acetate of D-ribonic acid when acted on by phosphorus pentachloride gives D-ribonyl chloride tetra-*O*-acetate. This latter compound can be converted into *keto*-D-psicose penta-*O*-acetate by reaction with diazomethane in ether and subsequent treatment of the 1-diazo-1-deoxy-*keto*-D-psicose tetra-*O*-acetate so formed, with copper acetate and acetic acid.[255]

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[249] J. Honeyman, *J. Chem. Soc.* **1946,** 990.

[250] S. Mukherjee and A. R. Todd, *J. Chem. Soc.* **1947,** 969.

[251] P. W. Kent, M. Stacey, and L. F. Wiggins, *J. Chem. Soc.* **1949,** 1232; *Nature* **161,** 21 (1948).

[252] R. Allerton and W. G. Overend, *J. Chem. Soc.* **1951,** 1480.

[253] C. F. Huebner, R. Lohmar, R. J. Dimler, S. Moore, and K. P. Link, *J. Biol. Chem.* **159,** 503 (1945).

[254] R. A. Baxter, A. C. McLean, and F. S. Spring, *J. Chem. Soc.* **1948,** 523.

Soon after the first preparation of D-ribose, Fischer[256] succeeded in reducing ribonolactone through the free sugar to a polyol (ribitol, adonitol). Catalytic reduction with Raney nickel of *aldehydo*-D-ribose 2,3,4,5-tetra-*O*-acetate afforded 2,3,4,5-tetra-*O*-acetylribitol.[257] A method has been patented for the hydrogenation of sugars to the corresponding polyol by magnesium-activated Raney nickel and in this way ribose can be converted to ribitol practically quantitatively.[258]

The condensation of nitromethane with D-ribose has been studied by Sowden and Fischer,[84, 259] and the results have been discussed in a recent extensive review.[260] Like other pentoses D-ribose is converted to furfural when heated with dilute acid: quantitative aspects of the conversion have been investigated.[261] The behavior of ribose in the Kiliani-Fischer synthesis has been reviewed by Hudson[262] (see also Richtmyer[81]).

IV. Chemistry of 2-Deoxyribose

1. PREPARATION

The isolation of 2-deoxy-D-ribose from deoxyribonucleic acid has proved to be very difficult. In early experiments it was usual to degrade the nucleic acid by chemical methods to the constituent nucleosides, separate these, and then hydrolyze the glycosidic linkage and isolate the sugar portion. Separation of the nucleosides was a tedious procedure, and, since acidic treatment for hydrolysis results in the conversion of some of the deoxypentose to levulinic acid, yields were poor. Following attempts by Thannhauser and Ottenstein,[263] who employed picric acid for the hydrolysis of thymus nucleic acid and obtained diphosphoric esters of pyrimidine deoxyribosides, Levene and London[264] resorted to enzymic methods of degradation and isolated deoxyribonucleosides of guanine, hypoxanthine (arising from deamination of adenine), cytosine and thymine. Very mild hydrolysis of the guanine nucleoside gave the deoxysugar in crystalline form.[265, 266]

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[255] M. L. Wolfrom, A. Thompson, and E. F. Evans, *J. Am. Chem. Soc.* **67**, 1793 (1945).

[256] E. Fischer, *Ber.* **26**, 633 (1893).

[257] H. H. Fox, *J. Org. Chem.* **13**, 580 (1948).

[258] L. A. Flexser, U. S. Pat. 2,421,416 (June 3, 1947).

[259] J. C. Sowden and H. O. L. Fischer, U. S. Pat. 2,480,785 (Aug. 30, 1949).

[260] J. C. Sowden, *Advances in Carbohydrate Chem.* **6**, 291 (1951).

[261] R. C. Hockett, A. Guttag, and M. E. Smith, *J. Am. Chem. Soc.* **65**, 1 (1943).

[262] C. S. Hudson, *Advances in Carbohydrate Chem.* **1**, 1 (1945).

[263] S. J. Thannhauser and B. Ottenstein, *Z. physiol. Chem.* **114**, 17, 39 (1921).

[264] P. A. Levene and E. S. London, *J. Biol. Chem.* **83**, 793 (1929).

[265] P. A. Levene and T. Mori, *J. Biol. Chem.* **83**, 803 (1929).

[266] P. A. Levene, L. A. Mikeska, and T. Mori, *J. Biol. Chem.* **85**, 785 (1930).

Similar mild acidic hydrolysis of hypoxanthine deoxyriboside gave a solution with the same optical rotation as an equivalent amount of 2-deoxy-D-ribose.[264] Attempts to isolate the sugar from the pyrimidine deoxyribonucleosides were unsuccessful, as the sugar was immediately converted into levulinic acid under the more drastic hydrolytic conditions necessary to cleave the linkage between the pyrimidine base and the deoxysugar.[264] Development of an improved enzymic hydrolysis procedure[267] and of chromatographic and ion-exchange methods[268-272] for the separation of deoxyribo-nucleotides and -nucleosides has afforded the possibility of obtaining these products in good yield in a high degree of purity. The writers and colleague[273] have succeeded in developing a method for obtaining 2-deoxy-D-ribose in fair yield by acidic hydrolysis of purine deoxyribonucleosides which had been separated by ion-exchange resin chromatography. Enzymic evidence has been provided[274] to show that deoxyribonucleotides isolated by the above methods are esterified at carbon atom 5 of the sugar moiety. Consequently, there is also the possibility that in the near future 2-deoxy-D-ribose-5-phosphate will be obtainable from nucleic acid.

Kent[274a] demonstrated that mercaptanolysis of deoxyribonucleic acids resulted in the liberation of the sugar which was isolated as the dibenzyl mercaptal.

The best known and probably still the most direct method for the synthesis of 2-deoxy-D-ribose is the "glycal" method, which is a general method for the synthesis of 2-deoxysugars. In this reaction, the elements of water are added to the olefinic linkage in a glycal by treatment with dilute sulfuric acid at low temperature and in this way D-arabinal (X) has been converted into 2-deoxy-D-ribose.[275-279] Likewise the conversion has been effected in the L-series.[74, 266, 277, 280] If the glycal is treated with a 2–3 % solution of hydrogen chloride in methanol instead of with dilute aqueous acid, then the methyl glycoside of the 2-deoxysugar is obtained. Fo example, L-arabinal was converted into methyl 2-deoxy-β-L-ribopyrano side.[281] The reactions involved in the glycal synthesis have been studied i considerable detail, especially in the conversion of arabinose to arabina and then into 2-deoxyribose, and the overall yield of this particular con version has been doubled by recently introduced improvements, but it i still very low.[277]

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[267] W. Klein, *Z. physiol. Chem.* **218,** 164 (1933).
[268] O. Schindler, *Helv. Chim. Acta* **32,** 979 (1949).
[269] P. Reichard and B. Estborn, *Acta Chem. Scand.* **4,** 1047 (1950).
[270] E. Volkin, J. X. Khym, and W. E. Cohn, *J. Am. Chem. Soc.* **73,** 1533 (1951).
[271] R. L. Sinsheimer and J. F. Koerner, *Science* **114,** 42 (1951).
[272] W. Andersen, C. A. Dekker, and A. R. Todd, *J. Chem. Soc.* **1952,** 2721.
[273] S. G. Laland and W. G. Overend, *Acta Chem. Scand.*, **8,** 192 (1954).
[274] C. E. Carter, *J. Am. Chem. Soc.* **73,** 1537 (1951).
[274a] P. W. Kent, *Nature* **166,** 442 (1950).
[275] G. E. Felton and W. Freudenberg, *J. Am. Chem. Soc.* **57,** 1637 (1935).
[276] A. M. Gakhokidze, *Zhur. Obshcheĭ Khim.* **15,** 539 (1945).
[277] R. E. Deriaz, W. G. Overend, M. Stacey, Ethel G. Teece, and L. F. Wiggins, *J. Chem. Soc.* **1949,** 1879.
[278] K. Ohta, *J. Biochem. (Japan)* **38,** 31 (1951).
[279] K. Ohta and K. Makino, *Science* **113,** 273 (1951).
[280] J. Meisenheimer and H. Jung, *Ber.* **60,** 1462 (1927).

2-Deoxy-D-ribose has been prepared by using D-erythrose or its derivatives as the initial material.[5, 6] Sowden[5] used both 2,4-O-benzylidene-D erythrose and D-erythrose as initial materials. The former was condense with nitromethane and sodium methoxide to give a mixture of 3,5-O benzylidene-1-nitro-1-deoxy-D-ribitol and -D-arabitol; these were separate by chloroform extraction. Hydrolysis and acetylation of the arabito derivative resulted in the formation of 2,3,4,5-tetra-O-acetyl-1-nitro-1 deoxy-D-arabitol, which was converted into D-*erythro*-triacetoxy-1-nitro pentene by boiling under reflux in benzene solution with sodium bicarbon ate. Similarly this compound could be prepared from D-erythrose.[5, Reduction of the pentene derivative, followed by treatment with sulfuri acid yielded 2-deoxy-D-ribose. Sowden[5] purified the product by formin the benzylphenylhydrazone and regenerated the deoxypentose by treatin with either benzaldehyde or formaldehyde. Overend and co-workers favored formation of the aniline derivative ("anilide") as a means of isolation and purification. Deanilination was effected by treatment with 0.5 % oxalic acid in aqueous solution, and 2-deoxy-D-ribose was obtained i crystalline form. Good yields are obtained at all stages of this synthesis and for preparative purposes Sowden[5] claims that the isolation of intermediates is unnecessary. The method would be a valuable one for th preparation of 2-deoxy-D-ribose, if D-erythrose was obtainable in a pur state in large quantities.

Attempts have been made to convert methyl 2,3-anhydroriboside (XLIV) int methyl 2-deoxy-D-riboside. The anhydro ring in XLIV can theoretically cleave i two ways, giving rise to two different products, i.e., a 2-substituted derivative o methyl D-arabinoside (XLV) and a 3-substituted derivative of methyl D-xylosid (XLVI). By using appropriate reagents for the cleavage it is possible to obtain derivatives which can be converted directly to 2- or 3-deoxysugar derivatives. Th protection afforded by the glycosidic residue increases the possibility of improve overall yields of the deoxysugars.

Methyl 2,3-anhydro-β-L-ribopyranoside was caused to react with sodium methy mercaptide and thereafter the product was boiled under reflux with Raney nick to effect catalytic desulfurization of the mixture of methyl 2-methylthio-2-deoxy β-L-arabinoside (XLV, R = SMe, D-isomer) and methyl 3-methylthio-3-deoxy- L-xyloside (XLVI, R = SMe, D-isomer). The main deoxypentoside obtained wa

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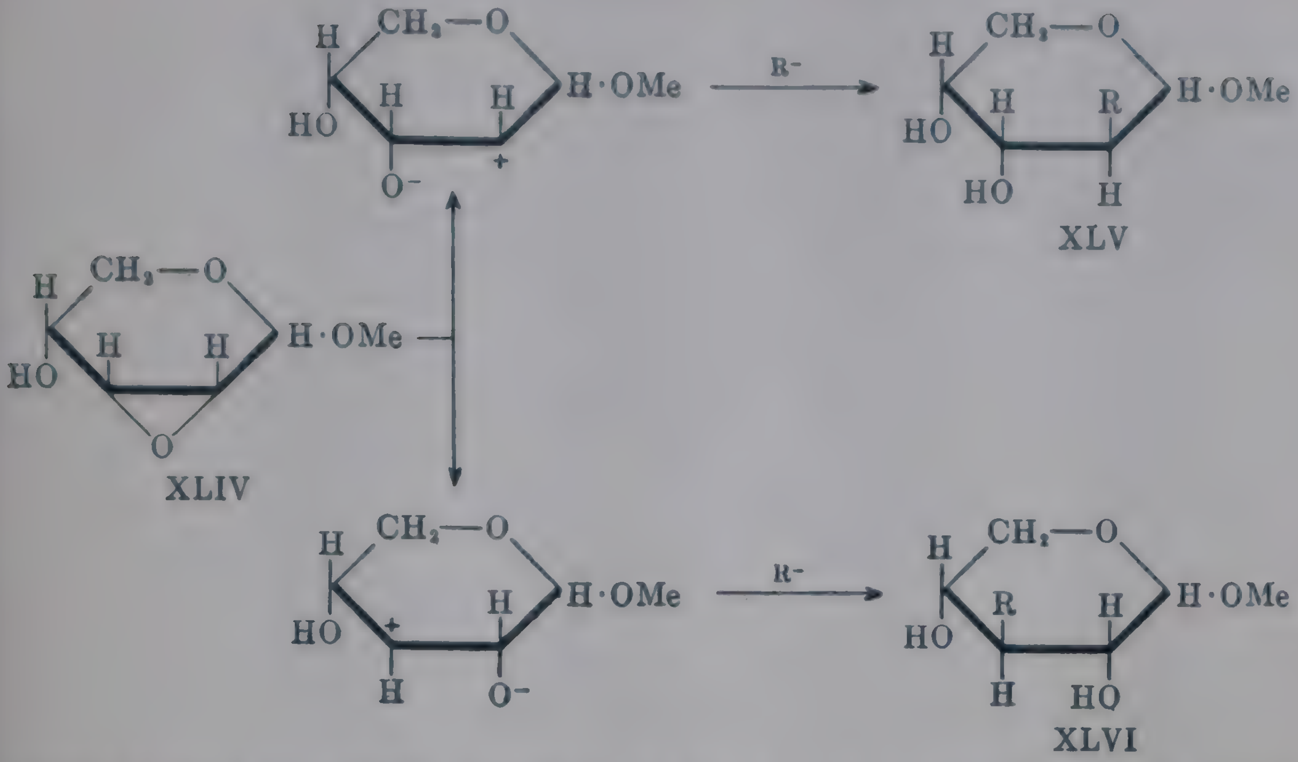
[281] R. E. Deriaz, W. G. Overend, M. Stacey, and L. F. Wiggins, *J. Chem. Soc.* **194** 2836.



methyl 3-deoxy-β-L-riboside (xyloside) (XLVI, R = H, D-isomer), and only traces of methyl 2-deoxy-β-L-riboside (XLV, R = H, D-isomer) were detected.[250] Change of configuration at the glycosidic center had no effect in increasing the proportion of the 2-deoxypentose derivative, since similar results were obtained with methyl 2,3-anhydro-α-L-ribopyranoside.[250] Likewise change in the size of the lactol ring had no effect on the orientation of the substituents.[282]

The action of halogen acids on alkyl 2,3-anhydroaldosides results in the formation of an alkyl 2-halogeno- or 3-halogeno-deoxyaldoside which can readily be reduced to give the corresponding alkyl 2- or 3-deoxyaldoside. Methyl 2,3-anhydro-β-D-riboside when treated with hydrobromic acid yielded mainly methyl 3-bromo-3-deoxy-β-D-xyloside (XLVI, R = Br) and only a small amount (10%) of methyl 2-bromo-2-deoxy-β-D-arabinoside (XLV, R = Br). After separation of these isomers, the methyl 2-bromo-2-deoxy-β-D-arabinoside was subjected to catalytic hydrogenation with Raney nickel in the presence of calcium hydroxide and afforded methyl 2-deoxy-D-ribose. This on hydrolysis with dilute acetic acid yielded 2-deoxy-D-ribose which was isolated as N-phenyl-2-deoxy-D-ribosylamine. Allerton and Overend[252] showed that the action of hydrochloric acid on this anhydroriboside leads to a slightly better yield (18.4%) of the methyl 2-halogeno-2-deoxy-D-arabinoside (XLV, R = halogen), but these methods have no value for the large-scale synthesis of 2-deoxy-D ribose.

Attempts to convert methyl anhydroriboside directly into methyl 2-deoxyriboside have not been very successful. Reaction of methyl 2,3-anhydro-β-D-riboside with lithium aluminum hydride gave mainly methyl 3-deoxy-β-D-riboside and only a small amount (14%) of methyl 2-deoxy-β-D-riboside.[252] On heating methyl 2,3-anhydro-β-L-riboside at 110° in hydrogen (at 100 atmospheres) with Raney nickel cleavage of the anhydro ring occurred to yield predominantly methyl 3-deoxy-β-L-riboside, accompanied by only a small amount of the 2-deoxy-isomer.[250]

Recently Allerton and Overend[283] succeeded in directly replacing by hydrogen the p-toluenesulfonyloxy (or methanesulfonyloxy) group in

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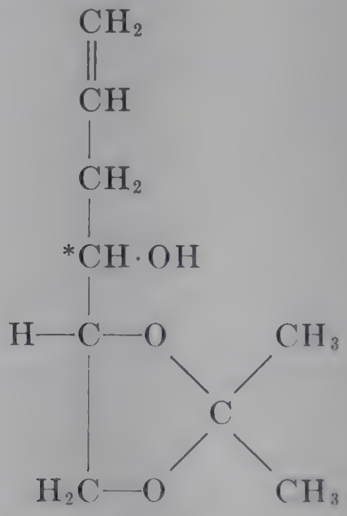
[282] J. Davoll, B. Lythgoe, and S. Trippett, *J. Chem. Soc.* **1951,** 2230.

[283] R. Allerton and W. G. Overend, *J. Chem. Soc.*, in press.

TECHNOLOGICAL RESEARCH

methyl 2-*O*-*p*-toluenesulfonyl(or methanesulfonyl)-β-L-arabinoside. The complex mixture of products contained some methyl 2-deoxy-β-L-riboside. Since derivatives of 3-deoxy-D-glucose became available, efforts have been directed towards the preparation of 2-deoxy-D-ribose from this deoxyhexose and from either calcium or barium 3-deoxy-D-gluconate. It has been shown in the writers' laboratory that when the modification of Ruff's method that was introduced by Hockett and Hudson[82] is employed, calcium 3-deoxy-D-gluconate can be degraded to 2-deoxy-D-ribose. Richards[284] has demonstrated that the application of Ruff's method of degradation to calcium *dextro*-metasaccharinate[285] under the conditions described by Fletcher *et al.*[286] affords the 2-deoxy-D-ribose in satisfactory yield. Furthermore, it is not necessary to separate α- from β-metasaccharinic acid, since a mixture of the two is equally effective. Hough[287] has outlined a novel method for the synthesis of 2-deoxy-D-ribose. An excess of allylmagnesium bromide was allowed to react with 2,3-isopropylidene-D-glyceraldehyde and, after decomposition of the resultant complex, syrupy 5,6-isopropylidene-1-hexene-4,5,6-triol (XLVII) was obtained in excellent yield. This reaction results in the formation of a new asymmetric center* at carbon atom 4. On treatment of XLVII with a solution of hydrogen peroxide in *tert*-butyl alcohol containing a little osmium tetroxide as catalyst, the double bond is hydroxylated and a new center of asymmetry is produced at carbon atom 2. From the mixture of products a fraction containing 5,6-*O*-isopropylidene-3-deoxyhexitols was obtained. Oxidation of this fraction with sodium metaperiodate destroyed the asymmetric center at carbon atom 2 and gave a mixture of 4,5-isopropylidene-2-deoxypentoses which on acidic hydrolysis



XLVII

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[284] G. N. Richards, *Chemistry & Industry* **1953,** 1035.

[285] J. U. Nef, *Ann.* **376,** 1 (1910).

[286] H. G. Fletcher, Jr., H. W. Diehl, and C. S. Hudson, *J. Am. Chem. Soc.* **72,** 4546 (1950).

[287] L. Hough, *Chemistry & Industry* **1951,** 406; *J. Chem. Soc.* **1953,** 3066.

yielded the free sugars. The major component was 2-deoxy-D-ribose and this was isolated as *N*-phenyl-2-deoxy-D-ribosylamine, from which the parent sugar was easily regenerated. Another method for the preparation of 2-deoxy-D-ribose, using 2,3-isopropylidene-D-glyceraldehyde as an initial material has been outlined briefly by Overend and Stacey.[288] The glyceraldehyde derivative was condensed with acetaldehyde in the presence of anhydrous potassium carbonate and the products were subjected to mild acidic hydrolysis to yield 2-deoxy-D-ribose and 2-deoxy-D-xylose. D-Arabinose has been converted into 2-deoxy-D-ribose in 3 % overall yield by the following sequence of reactions:[289] by heating in pyridine D-arabinose was converted into ribulose which was isolated as its nitrophenylhydrazone and reduced as such with Raney nickel catalyst to the 2-amino-2-deoxypentitols. The amino-alcohols were converted into the deoxypentose by treatment with nitrous acid. 2-Deoxy-D-ribose was isolated as its benzylphenylhydrazone.

2. IDENTIFICATION

In addition to the usual methods of carbohydrate identification, and the preparation of suitable derivatives (anilide, benzylphenylhydrazone, etc.), several color tests are available to test for this deoxypentose. Methods of identification of this sugar have recently been reviewed thoroughly by the authors.[4]

Furthermore, many of the color tests used to identify deoxyribonucleic acid depend on the sugar component of the nucleic acid. These color tests are described in Chapter 9. With the Dische[290] diphenylamine reagent, 2-deoxyribose gives an intense blue coloration. The test is not specific for 2-deoxyribose, but is given by 2-deoxypentoses generally[291, 292] and depends upon conversion of the 2-deoxysugar under acidic conditions into ω-hydroxylevulinaldehyde, which reacts with diphenylamine to give a blue-colored dyestuff.

3-Deoxy- and 2,3-dideoxyribose (but not 4-deoxyribose) also give faint blue colors with the diphenylamine reagent, but in these cases it is necessary to heat for a longer time than is required for 2-deoxyribose. The tryptophane reaction, introduced by Cohen[293] for the detection and estimation of deoxyribonucleic acids, is given equally well by 2-deoxyribose. In the recommended procedure, the material is heated at 100° for 10 minutes wit tryptophane and 30% (final concentration) perchloric acid, and in positive test there is a rapid development of a red color. Quantitative estimations can be carried out by measuring the intensity of the color developed in a photoelectric colorimeter with filters having a transmissio range of 485–550 mμ.

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[288] W. G. Overend and M. Stacey, *J. Sci. Food Agr.* **1,** 168 (1950).

[289] Y. Matsushima and Y. Imanaga, *Nature* **171,** 475 (1953); *Bull. Chem. Soc.* (Japan) **26,** 506 (1953).

[290] Z. Dische, *Mikrochemie* **8,** 4 (1930).

[291] R. E. Deriaz, M. Stacey, Ethel G. Teece, and L. F. Wiggins, *Nature* **157,** 740 (1946); *J. Chem. Soc.* **1949,** 1222.

[292] W. G. Overend, F. Shafizadeh, and M. Stacey, *J. Chem. Soc.* **1950,** 1027

[293] S. S. Cohen, *J. Biol. Chem.* **156,** 691 (1944).

The intensities of the colors produced by normal hexoses and pentose and their 2-deoxy-analogues with Schiff's reagent[294] have been measure quantitatively[295] with strict control of temperature and air contamination It was found that 2-deoxyribose gave a much more intense color tha ribose under comparable conditions. Gurin and Hood[296] have demonstrated that addition of a solution of carbazole to an ice-cold mixture o sulfuric acid and 2-deoxyribose results in the formation of an intense yello color. This test, however, is not as suitable as the diphenylamine reaction for the detection and estimation of 2-deoxyribose and is markedly unspecific. The reaction of cysteine and sulfuric acid[297] with 2-deoxyribose has also been used for estimation purposes. [Cf. *Dische*, Chapter 9.] Only aldehydes with an α-methylene group (i.e., R—CH_2CHO) condense with 3,5-diaminobenzoic acid to form quinaldines.[298] Consequently, in the carbohydrate series only 2-deoxysugars will react and hence they can be differentiated from their normal parent sugars. Attempts have been made to estimate 2-deoxyribose by the orcinol reaction for pentoses, but in the test the deoxysugar is mainly converted into levulinic acid which gives no color with the reagent. The Dreywood[299] anthrone reagent, which gives a positive qualitative test for a large variety of carbohydrates, gives a negative test for 2-deoxyribose.[300]

Recently, color tests have been found by which deoxysugars may be distinguished from other sugars or sugar derivatives on paper chromatograms.[301] Partridge[131] has reported R_F values (corrected to 20°) of 2-deoxy-D-ribose on Whatman No. 1 filter paper in various solvents. These were as follows:

Solvent........	Phenol containing NH_3 (1% wt./vol.) : HCN	s-Collidine	Isobutyric acid
R_F value........	0.73	0.60	0.32

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[294] H. Schiff, *Ann.* **140,** 102 (1866).

[295] W. G. Overend, *J. Chem. Soc.* **1950,** 2769.

[296] S. Gurin and Dorothy B. Hood, *J. Biol. Chem.* **131,** 211 (1939); **139,** 775 (1941)

[297] Z. Dische, *Proc. Soc. Exptl. Biol. Med.* **55,** 217 (1944).

[298] L. Vellu, M. Pesez, and G. Amiard, *Bull. soc. chim. France* **15,** 680 (1948).

[299] R. Dreywood, *Ind. Eng. Chem., Anal. Ed.* **18,** 499 (1946).

[300] L. Sattler and F. W. Zerban, *J. Am. Chem. Soc.* **72,** 3814 (1950).

[301] J. T. Edward and Deirdre M. Waldron, *J. Chem. Soc.* **1952,** 3631.

3. Physical Properties

Deriaz *et al.*[277] investigated the mutarotation in aqueous solution of 2-deoxyribose. The velocity constants for the mutarotations of both the D- and L-forms of this sugar were calculated, and the values obtained were 2 to 3 times greater than those for L-arabinose. In the presence of 0.01 N hydrochloric acid or 0.01 N sodium hydroxide, 2-deoxyribose reached the equilibrium value for the specific rotation immediately on dissolution. Values of the physical constants of 2-deoxyribose and its derivatives are listed in the Appendix (Table VIII.)

4. Properties and Reactions of Derivatives

a. O-Glycosides

Early in the development of deoxysugar chemistry it was noted that the rates of formation and hydrolysis of deoxysugar glycosides greatly exceeded those of the corresponding normal pentose or hexose. When 2-deoxy-L-ribose was treated with 1% methanolic hydrogen chloride it afforded a methyl 2-deoxyriboside mixture which was separated into crystalline α- and β-isomers (A and B, respectively).[281] If 0.1% methanolic hydrogen chloride was used then a third methyl 2-deoxypentoside (C) was obtained. The glycoside C was much more rapidly hydrolyzed by acids than were the glycosides A and B,[281] thereby indicating that C was probably a glycofuranoside. The glycosides A, B and C were separately mechanically shaken with acetone and anhydrous copper sulfate. Glycosides A and B readily formed mono-*O*-isopropylidene derivatives whereas C was recovered unchanged.[252] Since it is usual for acetone to condense with adjacent *cis*-hydroxyl groups, this would imply that A and B had pyranose structures and C a furanose structure.

When the glycosides B and C were treated with *p*-toluenesulfonyl chloride in dry pyridine, they both yielded a di-*p*-toluenesulfonyl derivative. That from the glycoside C readily underwent exchange with one mole of sodium iodide when heated at 105–110° for 3 hours with excess sodium iodide in acetone, whereas the derivative from B was unaffected by this treatment, indicating that only in glycoside C was there a primary hydroxyl group. From this it follows that B had a pyranose and C a furanose structure. Results of oxidation of the glycosides A, B and C with lead tetraacetate confirmed these conclusions.[281] Final proof of the structures of the glycosides B and C was obtained by methylation, hydrolysis, oxidation and comparison of the rates of hydrolysis of the lactones so obtained. From the glycoside B a 1,5-lactone was obtained[281] whereas a 1,4-lactone was derived from C, showing that the glycoside B had a 1,5-pyranose lactol ring and C a 1,4-furanose lactol ring. Since A and B were α- and β-anomers, it followed that A also had a pyranose structure.

Independent proof of the structure of B was furnished by the fact that it could be obtained from methyl 2,3-anhydro-β-D-ribopyranoside by a series of transformations which did not affect the lactol ring structure or configuration at carbon atom 1 of the initial material or intermediates in the conversion.[251] Further, this synthesis served to establish the α,β-relationship in the pyranose series, since the product was the optical enantiomorph of the glycoside B designated as methyl 2-deoxy-β-L-ribopyranoside. Davoll and Lythgoe[302] have pointed out that configurational assignments for methyl deoxyglycosides cannot be based only on optical rotation measurements and application of Hudson's[303] system that the more dextrorotatory anomer of a D-compound is termed α-D-, the other anomer being the β-D form, since it is not certain that the contribution of the asymmetric center at carbon atom 1 will be of the same sign in methyl 2-deoxyglycosides as in the related methyl glycosides. The α,β-relationship between methyl 2-deoxyglycosides was studied by Stacey and his colleagues.[281] They showed that 1% methanolic hydrogen chloride separately converts the α- and β-isomers of methyl 2-deoxy-L-ribopyranoside into the α,β-mixture from which they were initially isolated.

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[302] J. Davoll and B. Lythgoe, *J. Chem. Soc.* **1949**, 2526.

An outstanding property of the *O*-glycosides of 2-deoxysugars is the lability of the glycosidic substituent towards acid. Calculations from the experimental data of the velocity constant (*K*) for the hydrolysis of the methyl glycosides of D-arabinose and 2-deoxy-L-ribose (i.e., 2-deoxy-L-arabinose), with 0.01 *N* hydrochloric acid at 100°, gave the following results:[304]

(1) D-*Arabinose:*

Methyl glycoside	α-pyranoside	β-pyranoside	α,β-furanoside
$K\left[=\frac{1}{t}\log\frac{r_0 - r_\infty}{r - r_\infty}\ (t \text{ in minutes})\right]$	0.00064	0.00064	0.0068

(2) *2-Deoxy*-L-*ribose:*

Methyl glycoside	α-pyranoside	β-pyranoside	α,β-furanoside
K	0.18	0.22	1.4

It was noted by Stacey and co-workers[281] that, on distillation of methyl 2-deoxy-α,β-L-ribofuranoside, polymeric material was formed whenever cautious superheating occurred.

b. *N-Glycosides*

N-Phenyl-2-deoxy-D-[6] and -L-ribosylamine[277] have been obtained in crystalline form by the usual methods of synthesis, and are useful for the isolation and characterization of this deoxypentose. It is thought that these derivatives have a pyranose (1,5)-lactol ring structure; the stereochemical configuration of the sugar-base linkage is unknown. *N*-Glycosides of 2-deoxysugars are unable to undergo the Amadori rearrangement, and it is considered likely that the specificity of the reaction of deoxyribose with secondary amines to yield colored products is a function of the inability of 2-deoxysugar derivatives to participate in this rearrangement.

Although deoxyribonucleosides have been isolated from natural sources, attempts to synthesize them have been less successful. The reaction investigated was the coupling of an acetyl(or benzoyl)-1-bromo-2-deoxysugar with the silver salt of a base. Attempts to prepare 3,4-di-*O*-benzoyl-2-deoxy-D-ribopyranosyl bromide by treating syrupy 1,3,4-tri-*O*-benzoyl-2-deoxy-D-ribose with hydrogen bromide in acetic acid resulted in extensive decomposition. Treatment of syrupy 1,3,4-tri-*O*-acetyl-D-ribose with ethereal hydrogen chloride also resulted in decomposition, although in this case condensation of the crude product with theophylline silver gave a very small quantity of 3′,4′-di-*O*-acetyl-2′-deoxy-D-ribopyranosyltheophylline (D).[302]

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[303] C. S. Hudson, *J. Am. Chem. Soc.* **31,** 66 (1909).

[304] T. Reichstein, *Angew. Chem.* **63,** 412 (1951).

Diacetyl-D-arabinal was treated with hydrogen chloride in benzene and theophylline silver added to the crude product. Two forms (3 and 23 %) of 3′,4′-di-*O*-acetyl-2′-deoxy-D-ribopyranosyltheophylline were obtained. [The isomer formed in the smaller amount, was identical with the product (D)]. Deacetylation afforded two forms of 2′-deoxy-D-ribopyranosyltheophylline, which were considered to be α- and β-anomers. To avoid instabilities inherent in the deoxysugar, Lythgoe and his colleagues[282] attempted to defer its formation until the final stage of the synthesis by making 2′,3′-anhydro-5′-*O*-trityl-7-β-D-ribofuranosyltheophylline and then converting it to the deoxysugar derivative by the procedures available for the conversion of sugar epoxide rings to deoxysugars, but a negligible amount of the 2′-deoxyribose derivative was obtained. Some chemical properties of the naturally occurring deoxyribonucleosides have been examined. Towards acidic hydrolysis, cytosine deoxyriboside exhibited the greatest stability.[305] As with the ribose nucleosides, it was found that purine deoxyribosides rotate the plane of polarized light in a *levo* direction, whereas pyrimidine nucleosides are *dextro*rotatory.[264]

Reaction between 5,6-dimethylbenzimidazole silver and 3,4-di-*O*-acetyl-2-deoxy-D-ribopyranosyl chloride in xylene solution at 100° gave 5,6-dimethylbenzimidazole-1-(3′,4′-di-*O*-acetyl-2′-deoxy-D-ribopyranoside), hydrolysis of which furnished 5,6-dimethylbenzimidazole-1-(2′-deoxy-D-ribopyranoside).[166] In like manner, benzimidazole-1-(2′-deoxy-D-riboside) was prepared.

As is the case with *O*-glycosides, the *N*-glycoside derivatives of 2-deoxysugars are hydrolyzed by acid much more rapidly than the normal pentose or hexose analogues. This has been demonstrated for *N*-phenyl-D-ribosylamine and -2-deoxy-D-ribosylamine.[160] Cohn and his colleagues[270] state that adenine-9-(2′-deoxy-D-ribofuranoside-5′-phosphate), when in 0.01 *N* hydrochloric acid at room temperature, liberated adenine at a rate of 2 % per hour, whereas adenine-9-(β-D-ribofuranoside-5′-phosphate) was unaffected by this treatment. 5,6-Dimethylbenzimidazole glycosides are much more stable than arylamine-*N*-glycosides, but again the 2-deoxysugar derivatives are more labile than the normal sugar derivatives. For example, 5,6-dimethylbenzimidazole-1-(2′-deoxy-D-ribopyranoside) is hydrolyzed by heating in a sealed tube with 6 *N* hydrochloric acid for 12 hours at 100°, whereas the corresponding 5,6-dimethylbenzimidazole-1-(D-ribopyranoside) undergoes no hydrolysis.[166]

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[305] F. Bielschowsky and Marianne Siefken-Angermann, *Z. physiol. Chem.*, **207**, 210 (1932).

c. Phosphates

Manson and Lampen[306] reported that they obtained phosphorolysis and arsenolysis of hypoxanthine deoxyriboside by enzyme preparations from calf thymus gland and rat liver. An acid-stable phosphate was isolated as a product of phosphorolysis. It was concluded that this ester was 2-deoxy-D-ribose-5-phosphate and that it was formed from 2-deoxy-D-ribose-1-phosphate by mutase action. [Cf. *Glock*, Chapter 22, and *Schlenk*, Chapter 24.] The same authors[307] obtained indications for the formation of 2-deoxy-D-ribose-1-phosphate during the phosphorolysis of thymidine. Reverse reactions have been demonstrated since it was shown that hypoxanthine deoxy-D-riboside was formed enzymically from deoxy-D-ribose-1-phosphate and hypoxanthine.[308] Similarly, guanine deoxyriboside could be formed by using guanine in place of hypoxanthine.[309] Manson and Lampen[310] also observed nucleoside synthesis with the enzyme found in cell-free extracts of *Escherichia coli.* Determination of the equilibrium constant indicates that the synthesis rather than the splitting of hypoxanthine deoxy-D-riboside is favored.[308]

By enzymic phosphorolysis of guanine deoxy-D-riboside Friedkin[308] was able to isolate the labile phosphate in the form of a crystalline cyclohexylamine salt. This was a salt of the sugar phosphate designated above as 2-deoxy-D-ribose-1-phosphate and used in the experiments on nucleoside synthesis. Several reasons are forwarded to support the structure assigned to this compound. The ester, which contains one mole of 2-deoxy-D-ribose per mole of phosphorus, is extremely acid-labile—50% of the phosphorus present is released as inorganic phosphate within 10–15 minutes upon hydrolysis at pH 4 at 23°. This lability of the phosphate ester linkage points to carbon atom 1 as the site of esterification, and indeed a free aldehyde group is also released upon hydrolysis at pH 4.

The phosphate resulting from the mutase action on 2-deoxy-D-ribose-1-phosphate was isolated as the barium salt. Hydolysis with *N* hydrochloric acid for 7 minutes liberated 45% of the organic-bound phosphate. Since the sugar phosphate was reducing and gave no formaldehyde on periodate oxidation, it was, in view of its formation from hypoxanthine deoxy-D-riboside, assumed to be 2-deoxy-D-ribose-5-phosphate.

Arsenolysis of hypoxanthine deoxy-D-riboside resulted in the formation of hypoxanthine and free 2-deoxy-D-ribose.[306] Probably the primary product is 2-deoxy-D-ribose-1-arsenate, which decomposes in aqueous media to 2-deoxy-D-ribose and arsenate ions.

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[306] L. A. Manson and J. O. Lampen, *Abstracts Papers 114th Meeting Am. Chem. Soc* 53C (1948); *J. Biol. Chem.* **191,** 95 (1951).

[307] L. A. Manson and J. O. Lampen, *Federation Proc.* **8,** 224 (1949).

[308] M. Friedkin, *J. Biol. Chem.* **184,** 449 (1950).

[309] M. Friedkin and H. M. Kalckar, *J. Biol. Chem.* **184,** 437 (1950).

[310] L. A. Manson and J. O. Lampen, *Federation Proc.* **9,** 397 (1950).

Allerton *et al.*[311] have succeeded in synthesizing some phosphoric acid esters of 2-deoxy-L-ribose. Phosphorylation of methyl 2-deoxy-β-L-ribopyranoside with diphenylphosphorochloridate afforded the crystalline 3,4-*bis*diphenyl phosphate. Hydrogenation of this compound in the presence of Adam's catalyst afforded the syrupy 3,4-diphosphoric acid derivative which was characterized by the formation of its acridine and cyclohexylamine salts. Very mild acidic hydrolysis resulted in cleavage of the glycosidic methyl group without simultaneous scission of the phosphate residues and in this way 2-deoxy-L-ribose-3,4-diphosphate was obtained. By a similar series of reactions 2-deoxy-L-ribose-3,5-diphosphate was prepared from methyl 2-deoxy-α,β-L-ribofuranoside. Furthermore 2-deoxy-L-ribose-3-phosphate and 2-deoxy-L-ribose-5-phosphate were prepared and isolated as salts.

2-Deoxy-D-ribose-5-phosphate may be involved in the biosynthesis of this deoxypentose. [Cf. *Glock*, Chapter 22.] The suggestion was made by Hough and Jones[312] that deoxypentoses may arise from the aldol-type condensation of acetaldehyde and glyceraldehyde. Racker[313] has deduced evidence which indicates a similar route for the enzymic synthesis of deoxypentose from triose phosphate and acetaldehyde. Extracts of *E. coli*, *C. diphtheriae* and *S. fecalis* prepared by sonic disintegration of the bacterial cells, or by grinding with alumina, are capable of catalyzing the reversible reaction:

$$\text{glyceraldehyde phosphate} + \text{acetaldehyde} \leftrightharpoons \text{deoxypentose phosphate}$$

By combining the enzyme (phosphodeoxyriboaldolase) bringing about this change with purified phosphoriboaldolase from yeast, Racker[313b] was able to demonstrate the long-sought conversion of D-ribose into deoxy-D-ribose; e.g.

$$\text{D-ribose-5-phosphate} \xrightarrow{\text{(2 enzymes)}} \text{deoxy-D-ribose-5-phosphate}$$

It is apparent that a triose phosphate is the common intermediate between D-ribose and 2-deoxy-D-ribose in metabolism.

d. *Other Derivatives and Reactions*

The action of acid on 2-deoxyribose results in the formation of ω-hydroxylevulinaldehyde and then levulinic acid.[291] Oxidation with bromine water follows the normal course and affords 2-deoxyribonic acid.[74, 276] Similarly, oxidation can be achieved with barium hypoiodite in the presence of barium hydroxide.[266] The acid can be converted to a lactone: in soluti 2-deoxy-L-ribonolactone undergoes no change in optical rotation duri 8 days.[277] Likewise 3,5-di-O-methyl-2-deoxy-L-ribonolactone underwe negligible hydrolysis, but 3,4-di-O-methyl-2-deoxy-L-ribonolactone w completely hydrolyzed in 96 hours.[281] When di-O-acetyl-D-arabinal is treat with chlorine in chloroform solution, it yields 1,2-dichloro-1,2-dideoxy- pentose 3,4-di-O-acetate, which on reacting with silver carbonate in eth is converted to 2-chloro-2-deoxypentose 3,4-di-O-acetate. Heating wi lead oxide in a mixture of chloroform and water results in simultaneous r arrangement and deacetylation of the latter compound and affords 2-d oxy-D-ribonolactone.[276]

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[311] R. Allerton, W. G. Overend, and M. Stacey, *Chemistry & Industry* **1952,** 952.
[312] L. Hough and J. K. N. Jones, *Nature* **167,** 180 (1951).
[313] E. Racker, (a) *Nature* **167,** 408 (1951); (b) *J. Biol. Chem.* **196,** 347 (1952).

Esters (e.g., 1,3,4-tri-O-acetate and 1,3,4-tri-O-benzoate) of 2-deox D-ribose have been obtained crystalline.[252] The deoxypentose forms a be zyl-[278, 279] and p-nitrophenyl-hydrazone,[252] but does not form osazones. affords mercaptal derivatives,[277] and methyl[281] and trityl[311] ethers of th sugar are also known.

V. Addendum

It has been demonstrated recently that D-ribose occurs in natural m terials additional to those already described. A study of the immunolog cally active type-specific substance of *Hemophilus influenzae*, type b, ha given indications that this material consists of a polyribophosphate chai as it exists in pentose nucleic acids, in which the place of the purines an pyrimidines is occupied by a second similar chain, linked to the first 1,1′-glycosidic linkages.[314]

Several nucleosides and nucleotides have been isolated. Nebularin a natural product which was isolated[315] from the mushroom, *Agaric (Clitocybe) nebularis* Batsch., and which gave purine and ribose on h drolysis[316] has been shown to be 9-β-D-ribofuranosylpurine.[317] Marrian claims to have a new adenine nucleotide which is adenosine-5′-tetrapho phate, and the isolation from yeast of a nucleotide containing both ribo and mannose is reported.[319] The properties of this latter nucleotide a consistent with those of a structure in which the terminal phosphate grou of guanosine-5′-pyrophosphate is joined to a mannosyl residue, (i.e., it guanosine diphosphate mannose).

[314] S. Zamenhof, Grace Leidy, Patricia L. Fitz Gerald, Hattie E. Alexander, and Chargaff, *Federation Proc.* **11,** 315 (1952); *J. Biol. Chem.* **203,** 695 (1953).

[315] L. Ehrenberg, H. Hedström, N. Löfgren, and B. Takman, *Svensk. Kem. Tidsk* **58,** 269 (1946).

[316] N. Löfgren, and B. Lüning, *Acta Chem. Scand.* **7,** 225 (1953).

[317] G. B. Brown and Virginia S. Weliky, *J. Biol. Chem.* **204,** 1019 (1953).

[318] D. H. Marrian, *Biochim. et Biophys. Acta* **13,** 278 (1954).

[319] E. Cabib and L. F. Leloir, *J. Biol. Chem.* **206,** 779 (1954).

Wyatt and Cohen[320] have demonstrated that deoxyribonucleic acids of bacteriophages T2, T4, and T6 of *Escherichia coli* contain no cytosine: instead the pyrimidine base, 5-hydroxymethylcytosine, is a component of these nucleic acids. Subsequently, Weed and Courtenay[321] succeeded in isolating a deoxyribonucleotide containing hydroxymethylcytosine from the deoxyribonucleic acid of *Escherichia coli* bacteriophage.

D-[1-C^{14}]-Ribose has been prepared.[322] 2-Deoxy-D-ribose was obtained by partial oxidation of 3-deoxy-D-glucose with sodium metaperiodate, followed by deformylation of the product by rendering the reaction mixture slightly alkaline.[323] The product was isolated as *N*-phenyl-2-doxy-D-ribosylamine.

The synthesis of *N*-(3,4-dimethylphenyl)-D-ribosylamine by a rearrangement method has been reported.[324] 9-β-D-Ribofuranosylpurine ("nebularine") has been synthesized[317] by condensation of the chloromercuri-derivative of purine and tri-*O*-acetyl-D-ribofuranosyl chloride according to the general method of Davoll and Lowy.[325] Alternatively, 6-chloro-9-β-D-ribofuranosylpurine was prepared and subjected to reductive dehalogenation to afford nebularine. Further syntheses of 5,6-dimethylbenzimidazole-α-D-ribofuranoside (α-ribazole) have been reported. 2-Nitro-4,5-dimethyl-*N*-(2′,3′-di-*O*-acetyl-5′-*O*-trityl-D-ribofuranosido)aniline was reduced to the corresponding 2-amino derivative, and this syrupy product in benzene solution was stirred with carbon disulfide and a base (i.e., barium hydroxide) to afford 5,6-dimethyl-2-sulfhydrylbenzimidazole-2′,3′-di-*O*-acetyl-5′-*O*-trityl-D-ribofuranoside. Desulfurization with nickel of this latter compound and removal of the acetyl and trityl residues gave 5,6-dimethylbenzimidazole-α-D-ribofuranoside (isolated as the picrate) but none of the β-isomer.[326] Condensation of 5,6-dimethylbenzimidazole in dry dioxane at 100° with tri-*O*-acetyl-D-ribofuranosyl chloride followed by deacetylation of the reaction product afforded 5,6-dimethylbenzimidazole-α- and -β-ribofuranoside.[327] The yields of products were 2% and 10%, respectively, based on the amount of tetra-*O*-acetyl-D-ribofuranose used for preparation of the acetochloro-ribose. α-Ribazole phosphate has been synthesized by phosphorylation of the 5′-*O*-trityl derivative of α-ribazole with either diphenyl- or dibenzyl-phosphorochloridate and subsequent removal of pr tecting groups. This phosphate has approximately the same biologic activity as α-ribazole.[328] Folkers and his colleagues[326] have prepared 5, dimethylbenzimidazole-2′-deoxy-D-ribopyranoside (isolated as the picrat by condensation of 5,6-dimethylbenzimidazole silver and 3,4-di-*O*-acety 2-deoxy-D-ribopyranosyl chloride.

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[320] G. R. Wyatt and S. S. Cohen, *Nature* **170,** 1072 (1952); *Ann. inst. Pasteur* **84,** 143 (1953); *Biochem. J.* **55,** 774 (1953).

[321] L. L. Weed and T. A. Courtenay, *J. Biol. Chem.* **206,** 735 (1954).

[322] Harriet L. Frush and H. S. Isbell, *J. Research Natl. Bur. Standards* **51,** 307 (1953).

[323] P. A. J. Gorin and J. K. N. Jones, *Nature* **172,** 1051 (1953).

[324] V. A. Konkova, *Zhur. Obshcheĭ Khim.* **22,** 1896 (1952).

[325] J. Davoll and B. A. Lowy, *J. Am. Chem. Soc.* **73,** 1650 (1951).

[326] Dorothea Heyl, Edith C. Chase, C. H. Shunk, Marjorie V. Moore, Gladys A Emerson, and K. Folkers, *J. Am. Chem. Soc.* **76,** 1355 (1954).

[327] A. W. Johnson, G. W. Miller, J. A. Mills, and A. R. Todd, *J. Chem. Soc.*, **1953,** 3061

The viewpoint that cytidylic acid "b" is cytidine-3′-phosphate, base on indirect evidence[329, 330] (i.e., solubility, ultraviolet absorption, and aci strength measurements), has been reinforced by the results of a compariso of the infrared spectra of the phosphates of cytidine and deoxycytidine. The spectra of the two 5′-phosphates are closely similar, as also are thos of deoxycytidine-3′-phosphate (synthesized chemically[331]) and one form cytidylic acid "b." The infrared spectrum of cytidylic acid "a" (in eithe of its isomorphic modifications[332]) is quite different from any of these The conclusion that cytidylic acid "b" is cytidine-3′-phosphate is furthe supported by a comparison of optical rotation and ultraviolet absorptio data in the cytidine and deoxycytidine phosphates series.[331] It follows tha uridylic acid "b" is uridine-3′-phosphate since it can be prepared from cytidylic acid "b" by deamination under conditions which preclude phosphoryl migration.[333] In connection with work on the identification o adenylic acids "a" and "b" as the 2′- and 3′-phosphoadenosines, respectively, [see *Baddiley*, Chapter 4], Cohn and his colleagues[334] have prepare and characterized pure ribose-2- and -3-phosphates and have compared thei properties with the compounds described by Levene and Harris.[179, 213, 2 By hydrolysis at 100° for 4 minutes of adenylic acids with a polystyrene sulfonic acid cation-exchange resin a mixture of ribose-2- and -3-phosphat was obtained.[334] This mixture, which consisted of 36 parts of the 2-isomer and 64 parts of the 3-isomer, was separated by ion-exchange chromatography with borate complexing, and by fractional crystallizatio of the brucine salts. Although separation by the ion-exchange method gav pure ribose-2- and -3-phosphate, the crystallization method afforded onl pure dibrucine ribose-3-phosphate hexahydrate and the pure correspondin salt of ribose-2-phosphate could not be obtained. The pure ribose phosphates were characterized by their optical and ion-exchange behaviors, i the presence and absence of borate, by the differences in susceptibility of their methyl pyranosides to periodate oxidation, and by the differences in their decomposition rates in alkali. The decreasing order of stability to alkali (0.01 *N* NaOH at 22°) is ribose-2-phosphate—which is scarcely attacked—ribose-3-phosphate, and ribose-5-phosphate—which is the most labile. Methods were described for the interconversion of the various salts of these phosphates. Comparison of the properties of the salts of the pure ribose-2- and -3-phosphates prepared by Cohn *et al.*[334] with those described by Levene and Harris[179, 213, 214] for preparations of ribose phosphates indicates that the latter workers were handling mixtures composed of about 20 parts of ribose-2-phosphate and 80 parts of ribose-3 phosphate. In the light of present knowledge it is clear that the experimental conditions employed by Levene and Harris could not have resulted in retention of isomeric integrity, but would be expected to result in the formation of mixtures. Consequently, the samples prepared by Cohn and his colleagues are the first pure preparations of ribose-2- and -3-phosphate. When ribose-2- or -3-phosphate is heated for 2 hours with Dowex 50 (H^+) resin or for 45 minutes with 0.1 *N* sulfuric acid it forms ribose-4-phosphate.[334] The yields ranged from 8 to 10% of theory, and not enough material was isolated to permit characterization of a solid salt of the compound. Khym *et al.*[334] prepared ribose-5-phosphate by treating adenosine-5′-phosphate with Dowex 50 (H^+) resin at 100° for 4 minutes. Thereafter the solution was cooled and more than 96% of the adenine and adenosine compounds were removed by filtration. The ribose phosphate (*ca.* 95% yield) was isolated in crystalline form as the barium salt. A fraction containing 70–80% of D-ribose-5-phosphate is obtained when xylose and adenosine triphosphate are incubated with pentose phosphate isomerase (from extracts of *Lactobacillus pentosus*).[335] A new simplified procedure for the isolation of deoxyribose-1-phosphate has been developed: it involves the phosphorolysis of thymidine in the presence of dicyclohexylammonium hydrogen phosphate, followed by a fractionation step with *n*-butanol–diethyl ether, which yields crystalline dicyclohexylammonium deoxyribose-1-phosphate after a single filtration.[336] Reaction between thymine and deoxyribose-1-phosphate in the presence of mammalian thymidine phosphorylase gives thymidine which can be isolated in crystalline form.[336]

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[328] E. A. Kaczka, Dorothea Heyl, W. H. Jones, and K. Folkers, *J. Am. Chem. So* **74,** 5549 (1952).

[329] H. S. Loring, Myrtle L. Hammell, L. W. Levy, and H. W. Bortner, *J. Biol. Chem* **196,** 821 (1952).

[330] L. F. Cavalieri, *J. Am. Chem. Soc.* **74,** 5804 (1952).

[331] A. M. Michelson and A. R. Todd, *J. Chem. Soc.* **1954,** 34.

[332] R. J. C. Harris, S. F. D. Orr, E. M. F. Roe, and J. F. Thomas, *J. Chem. Soc.* **195** 489.

[333] D. M. Brown, C. A. Dekker, and A. R. Todd, *J. Chem. Soc.* **1952,** 2715.

[334] J. X. Khym, D. G. Doherty, and W. E. Cohn, private communication.

The preparation and properties of 2,3,4-tri-*O*-benzoyl-*β*-D-ribose have been described,[337] and new benzoyl derivatives of D-ribofuranose and *aldehydo*-D-ribose have been outlined.[338] A preliminary X-ray study is reported[339] of the solid state transformation in tetra-*O*-acetyl-D-ribof anose (cf. Davoll *et al.*[237] and Farrar[237a]).

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[335] J. O. Lampen, *J. Biol. Chem.* **204,** 999 (1953).

[336] M. Friedkin and DeWayne Roberts, *J. Biol. Chem.* **207,** 257 (1954).

[337] H. G. Fletcher, Jr., and R. J. Ness, *J. Am. Chem. Soc.* **76,** 760 (1954).

[338] R. K. Ness, H. W. Diehl, and H. G. Fletcher, Jr., *J. Am. Chem. Soc.* **76,** 763 (1954).

The hydrolysis of tri-*O*-benzoyl-β-D-ribofuranosyl bromide gives only 2,3,5-tri-*O*-benzoyl-β-D-ribose as previously reported but also substance which is most probably 3,5-di-*O*-benzoyl-1,2-*O*-(1-hydro benzylidine)-α-D-ribose.[340] The compound designated by Weygand a Wirth[341] as 2,3,5-tri-*O*-benzoyl-D-ribose has been shown to be identi with 3,5-di-*O*-benzoyl-1,2-*O*-(1-hydroxybenzylidine)-α-D-ribose.

[339] A. L. Patterson and Barbara P. Groshens, *Nature* **173**, 398 (1954).

[340] R. K. Ness and H. G. Fletcher, Jr., *J. Am. Chem. Soc.* **76**, 1663 (1954).

[341] F. Weygand and F. Wirth, *Chem. Ber.*, **85**, 1000 (1952).

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TABLE VII

DERIVATIVES OF D-RIBOSE[a]

Compound	M.p., °C.	$[\alpha]_D$ (solvent)	Reference
β-D-Ribose	(a)—(b) 86 (c) 87 (d)—(e) 86–87 (f) 83–87 (g) 86–87 (h) 95 (i) 95 (j) 95 (k) 86–87 (l) 91–92 (m) 87 (n) 78–79	(a) −19.7° (W); (b) —; (c) −23.7° (W); (d) −23° (W); (e) −19.6° (W); (f) −17.5°; (g) −19.4° (W); (h) −21.5°; (i) —; (j) —; (k) −19.5° (W); (l) −20° (W); (m) −23.1 (after 1.5 min.) → −23.7° (after 300 min.) (W); (n) −38.2° (I) → −43.1° (E) (Py)	(a) 138 (b) 141 (c) 78 (d) 79 (e) 86 (f) 33 (g) 88 (h) 16 (i) 100 (j) 105 (k) 12 (l) 111 (m) 129 (n) 96
1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-	(a) 126–127 (b) 130–131	(a) +23.3 ± 2° (Py); (b) +44.2° (Ch)	(a) 246 (b) 244
3,4-di-*O*-acetyl-,1,2-ortho-*O*-acetate	77–78	+2.4° (Ch)	110
3,4-di-*O*-acetyl-,1,2-ortho-3′-*O*-acetoxyacetonyl-acetate	97–98	−11.6° (Ch)	102
1,5-di-*O*-acetyl-2,3-*O*-isopropylidene-	liquid (b.p. 119–121°/0.01 mm.)	—	220
1,2,3-tri-*O*-acetyl-5-*O*-trityl-	—	+5.05° (Et)	96
2,3,4-tri-*O*-acetyl-	(a) syrup (b) syrup	(a) −26.3° (W); (b) −26.3° (W)	(a) 88 (b) 90
2,3,4-tri-*O*-acetyl-5-*O*-benzoyl	liquid (b.p. 170° (b.T.)/10^{-4} mm.)	—	245
2,3,4-tri-*O*-acetyl-5-*O*-benzyl	liquid (b.p. 150° (b.T.)/10^{-3} mm.)	−4.2 ± 0.8° (Ch)	232
2,3,4-tri-*O*-acetyl-5-*O*-trityl-	syrup	−4° (Ch); +0.3° (M)	235

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[a] In Tables VII and VIII the following abbreviations are used: I = initial; E = equilibrium; C = corrected; S = sinter; D = decomposition; b.p. = boiling point; b.T. = bath temperature; A = acetone; B = benzene; Ch = chloroform; Et = ethanol; EtAc = ethyl acetate; HAc = acetic acid; M = methanol; Py = pyridine; W = water.

TABLE VII—*Continued*

Compound	M.p., °C.	$[\alpha]_D$ (solvent)	Reference
1,2,3,4-tetra-*O*-acetyl-	(a) 110 (b) 109–110 (c) 110 (d) 110 (e) 108–109 (f) 110 (g) 110	(a) −52° (Ch); (b) −56° (Ch); (c) —; (d) —; (e) −54.1°; (f) —; (g) −55.4° (M)	(a) 110 (b) 102 (c) 141 (d) 159 (e) 33 (f) 141 (g) 235
1,2,3,4-tetra-*O*-acetyl-(deriv. of α-D-ribose)	syrup	+50.7° (M)	235
1,2,3,5-tetra-*O*-acetyl-	(a) 82 (b) 82 (c) 58 (d) 57–58 (e) 56	(a) −15.4° (M); (b) −12.6° (Ch); −15.4° (M); (c) +20 ± 1° (Ch); (d) —; (e) −3.6° (M)	(a) 235 (b) 141 (c) 144 (d) 232 (e) 236
1,2,3,5-tetra-*O*-acetyl-(deriv. of α-D-ribose)	syrup	+78.7° (M)	235
2,3,4,5-tetra-*O*-acetyl-(deriv. of *aldehydo*-D-ribose)	(a) 100 (b) 101–102 (c) 100 (d) 98–99	(a) +3.0° → +17.4° (M); (b) −16.2° (A); (c) −38.1° (Ch); +2.9° (I) → +17.1° (24 hr.) (M); (d) —	(a) 235 (b) 239 (c) 238 (d) 169
5-*O*-benzoyl-, phenylhydrazone	159	—	245
2,3,4-tri-*O*-benzoyl-	(a) syrup (b) 113–114	(a) −27.9° (Ch); (b) −42° (Ch)	(a) 145 (b) 146
2,3,5-tri-*O*-benzoyl-	112–113	+68.4° (Ch)	244
1,2,3,4-tetra-*O*-benzoyl-	(a) — (b) 131	(a) −100° (Ch); (b) −102° (Ch)	(a) 146 (b) 111
5-*O*-benzyl-	—	−8.5 (Et)	232
1,2,3,5-tetra-*O*-benzoyl-	121–122	+17.0° (Ch)	244
2,3-*O*-isopropylidene-	(a) liquid (b.p. 110–117°/0.05 mm.) (b) syrup	(a) —; (b) −27.4° (A)	(a) 220 (b) 101
2,3-*O*-isopropylidene-1,5-di-*O*-tosyl	122–123	—	220
2,3-*O*-isopropylidene-5-iodo-5-deoxy-1-*O*-tosyl	120	+84.6° (Ch)	220
2,3-*O*-isopropylidene-5-nitro-5-deoxy-1-*O*-tosyl-	156	—	220

5-*O*-methyl-, *p*-bromophenylosazone	(a) 175 (b) 161–162	(a) −55.2° (Et: Py); (b) −48° (Et: Py)	(a) 175 (b) 220
2,4-di-*O*-methyl-	liquid (b.p. 132–136°/< 1 mm.)	−16.7° (aq. NH_4OH)	214
3,5-di-*O*-methyl-, phenylosazone	161	—	134
2,3,4-tri-*O*-methyl-	(a) 85–86 (b) 98–100	(a) −51.7° → −40.0° (W); (b) —	(a) 136 (b) 231
2,3,5-tri-*O*-methyl-	(a) liquid (b.p. 97° (b.T.)/0.01 mm.) (b) liquid (b.p. 90–92°/0.02 mm.) (c) liquid (b.p. 90–92/0.1 mm.)	(a) +41.4° (M); (b) —; (c) +39.3° (M); +40.6° (W); +43.4° (aq. NH_4OH)	(a) 134 (b) 210 (c) 220
1-*O*-trityl-	148	+33.8° (Ch); +26.5° (Py)	234
5-*O*-trityl-	(a) 125 (b) 123–125	(a) +12.1° (after 4 min.) → −9.9° (12 hr.) (Py); (b) +11.3° (after 5 min.) → −2.9° (E) (Py)	(a) 96 (b) 113
1,5-di-*O*-trityl-	225	+61.2° (B); +48.5° (Py)	234
1,5-di-*O*-trityl-, 2,3-di-*O*-acetyl-	285	+29.2° (Ch)	234
1,3(or 2)-di-*O*-trityl-	186	+37.4° (Ch)	234
1,3(or 2)-di-*O*-trityl-2(or 3),5-di-*O*-acetyl-	228	+54.2° (Ch)	234
1,3(or 2)-di-*O*-trityl-2(or 3)-*O*-acetyl-	203	+22.1° (Ch); +53.8° (Py)	234
1,3,5-tri-*O*-trityl-	292	+89° (Ch); +72.6° (Py)	234
1,3,5-tri-*O*-trityl-2-*O*-acetyl-	204	−91.4° (Ch)	234
benzylphenylhydrazone	(a) 125 (b) 127–128 (c) 125.5–126.5 (d) 127–128	(a) —; (b) —; (c) −39 ±2°; (d) −24.46° (Et)	(a) 74 (b) 5 (c) 188b (d) 12
p-bromophenylhydrazone	(a) 165–165.5 (b) 166–167 (c) 165 (d) 164–165 (e) 164 (f) 165 (g) 164 (h) 164 (i) 164	(a) —; (b) —; (c) —; (d) +10.3° (Et); (e) —; (f) —; (g) —; (h) — (i)—5.7	(a) 102 (b) 70 (c) 79 (d) 101 (e) 31 (f) 100 (g) 16 (h) 33 (i) 103
p-bromophenylosazone	180–185	—	103

TABLE VII—*Continued*

Compound	M.p., °C.	$[\alpha]_D$ (solvent)	Reference
diphenylmethane-dimethyl-dihydrazone	141–142	—	105
phenylosazone	(a) 159–160 (b) 163–164 (c) 165–165.5	(a) —; (b) —; (c) —	(a) 31 (b) 12 (c) 33
p-toluene sulfonylhydrazone	(a) 164 (b) 164–165 (D)	(a) +23° (I) → +14° (after 16 hr.) (Py); (b) —	(a) 85 (b) 104
oxime	141	+6.3°	78
Benzyl-β-D-ribofuranoside	95–96	−60.5° (W)	244
2,3,5-tri-*O*-benzoyl-	87–88	+14.9° (Ch)	244
Ethyl-β-D-ribopyranoside	92–93	−104° (W)	145
2,3,4-tri-*O*-benzoyl-	132–133	−83.9°	145
Methyl-β-D-ribopyranoside	(a) 83 (b) 83–84	(a) −105° (W); (b) −113.6° (W)	(a) 138 (b) 137
2,3,4-tri-*O*-acetyl-	syrup (b.p. 120°/0.05 mm.)	−17.4° (Ch)	110
2,3,4-tri-*O*-benzoyl-	(a) 109–110 (b) 108–110	(a) −69.5° (Ch); (b) —	(a) 145 (b) 146
2,3-*O*-isopropylidene-4-*O*-tosyl-	144–145	−114.9° (Et)	247
2,4-di-*O*-methyl- (impure)	liquid (b.p. 90–93°/< 1 mm.)	−48.7° (M); −47.4° (W)	214
2,4-di-*O*-methyl-, 3-phosphate	—	−17.2°	213
2,3,4-tri-*O*-methyl-	liquid (b.p. 54°/0.05 mm.)	−35° (W)	136
4-*O*-tosyl-	124	−40° (Ch)	247
Methyl-D-ribofuranoside	syrup (b.p. 150° (b.T.)/0.01 mm.)	+13.1° (M)	134
5-*O*-benzyl-	liquid (b.p. 95–100° (b.T.)/10^{-4} mm.)	−36 ± 2° (Ch)	232
2,3-*O*-isopropylidene-	liquid (b.p. 83–86°/0.05 mm.)	—	175
2,3-*O*-isopropylidene-5-*O*-methyl-	liquid (b.p. 62–65°/0.03 mm.)	—	220

2,3-*O*-isopropylidene-5-*O*-tosyl-	83–84	−35.5° (Et)	247
2,3,5-tri-*O*-methyl-	(a) liquid (b.p. 133° (b.T.)/15 mm.) (b) liquid (b.p. 68°/0.05 mm.)	(a) +59.1° (M); (b) —	(a) 134 (b) 220
2′-Naphthyl-1-thio-β-D-ribopyranoside			
2,3,4-tri-*O*-benzoyl-	—	−59° (Ch)	111
α-D-Ribopyranosyl bromide			
2,3,4-tri-*O*-benzoyl-	164–166	+78° (Ch)	146
β-D-Ribopyranosyl bromide			
2,3,4-tri-*O*-acetyl-	(a) 96 (b) 96 (c) 94.5–95.5 (d) 96	(a) −209.3° (Ch); (b) —; (c) −223.9° (Ch); (d) −209.3° (Ch)	(a) 110 (b) 254 (c) 102 (d) 141
2,3,4-tri-*O*-benzoyl-	(a) 150–154 (b) 151–153	(a) 202° (Ch); (b) −199° (Ch)	(a) 146 (b) 111
D-Ribofuranosyl bromide			
2,3,5-tri-*O*-acetyl-	syrup	dextrorotatry (Ch)	141
α-D-Ribopyranosyl chloride			
2,3,4-tri-*O*-benzoyl-	203–204	+60° (Ch)	146
β-D-Ribopyranosyl chloride			
2,3,4-tri-*O*-acetyl-	95	−169.6° (Ch)	141
2,3,4-tri-*O*-benzoyl-	162–163	−147° (Ch)	146
D-Ribofuranosyl chloride			
2,3,5-tri-*O*-acetyl-	syrup	*ca.* +40° (Ch)	141
D-Ribobenzimidazole	(a) *ca.* 190 (D); (b) 190; (c) 239; (D); (d) 191; (e) 189–191	(a) +21.6° (5% citric acid); (b) +22.5° (*N* HCl); (c) −50.4° (5% citric acid); (d) +23.5° (5% citric acid); (e) +22° (5% citric acid)	(a) 106 (b) 107 (c) 19 (d) 20 (e) 21
hydrochloride	(a) 200–202 (b) 196–198 (c) 201–203	(a) —; (b) —; (c) —	(a) 21 (b) 107 (c) 20
picrate	(a) 184–186 (b) 185–186 (c) 184–186	(a) —; (b) —; (c) —	(a) 21 (b) 107 (c) 20

TABLE VII—*Continued*

Compound	M.p., °C.	$[\alpha]_D$ (solvent)	Reference
1′,4′-anhydro-	82–83	−84.5° (Et)	253
1′,4′-anhydro-, picrate	120–125	—	253
1-(β-D-Ribopyranosyl)-5,6-dimethylbenzimidazole	250–251 (D)	—	166
2′,3′,4′-tri-*O*-acetyl-	155	−40.4° (Ch)	166
2′,3′,4′-tri-*O*-acetyl-, picrate	186–187	—	166
hydrochloride hemihydrate	229–230	—	166
1-(α-D-Ribofuranosyl)-5,6-dimethylbenzimidazole			
picrate	212–213	+9.1 ± 1° (Py)	34
1-(β-D-Ribofuranosyl)-5,6-dimethylbenzimidazole			
picrate	175–177	−24 ± 2° (Py)	34
N-Phenyl-D-ribopyranosylamine	(a) 125–127 (b) 114–116 (c) 112–114 (d) 119	(a) +63.4° → +48.6° (Py); (b) +62° (after 3 min.) → +50° (after 24 hr.) (Py), + 23° → +13° (M); (c) +24.3° → +9.4° (M); +60.2° (Py); (d) +60° (Py)	(a) 89, 157, 158 (b) 160 (c) (c) 161 (d) 159
2,3,4-tri-*O*-acetyl-	—	+29.4° (Ch)	159
N-Phenyl-D-ribofuranosylamine	(a) 138–140 (b) 123–124 (c) 133–134 (d) 126–127	(a) +176.5° → +156.6° (Py); (b) +182° (after 4 min.) → +52.3° (Py); +135° (after 13 min.) → +12° (M); (c) +176.4° (Py); +134.9° → +14.1° (M); (d) +180° (Py)	(a) 86, 89, 157 (b) 160 (c) 161 (d) 159
2,3,5-tri-*O*-methyl-	56.5	—	134
N-*p*-carboxyphenyl-D-ribopyranosylamine	129–130 (D)	+231° → +70.2° (Py)	87, 158

N-*o*-Chlorophenyl-D-ribopyranosylamine	152–153	+136° → +125° (Py)	87, 158
N-*m*-Hydroxy-*p*-methylphenyl-D-ribopyranosylamine	133–135° (D)	+116° → +32.4° (Py)	87, 158
N-*p*-Methylphenyl-D-ribopyranosylamine	(a) 102–103 (b) 123	(a) +53.2° (Py); (b) +60.2° (Py)	(a) 87, 158 (b) 161
N-*p*-Methoxyphenol-D-ribopyranosylamine	109–110	+122° → +40.8° (Py)	87, 158
N-(1-Naphthyl)-D-ribopyranosylamine	146–147	+122° → +29.2° (Py)	87, 158
N-(2-Naphthyl)-D-ribopyranosylamine	119–120	+96.6° (Py)	87
N-*o*-Nitrophenyl-D-ribopyranosylamine	(a) 183–185 (D); (b) 193–194	(a) −109° (Py); (b) −109.1° (Py)	(a) 87, 158 (b) 161
N-3,4-Dimethylphenyl-D-ribopyranosylamine	(a) 110–112 (b) 118	(a) +94.5 → +53.0 (Py); (b) +172° (Py)	(a) 157, 158 (b) 170
N-3,4-Dimethylphenyl-D-ribofuranosylamine	129–130	+171.7° → +56.5° (Py)	157, 158
N-*p*-Methylphenyl-D-ribofuranosylamine	130	+178.2° → +76° (Py)	161
N-*o*-Nitrophenyl-D-ribofuranosylamine	167–168	−122.5° (Py)	161
N-2-Nitro-4,5-dimethylphenyl-D-ribosylamine	164	+90 ± 3° (Py)	154
tri-*O*-acetate	163	+160 ± 5° (MeAc)	154
D-Ribose dimethyl mercaptal	(a) 76–76.5 (b) 78 (c) 76–76.5	(a) −9.4° (M); (b) −21° (W); −9.5° (Et); (c) −9.6 (M)	(a) 114a (b) 112 (c) 114b
2,3,4-tri-*O*-acetyl-5-*O*-trityl-	123–124	−2.3° (Ch)	113
2,3,4-tri-*O*-benzoyl-5-*O*-trityl-	—	+21.9 (Ch)	113
5-*O*-trityl-	—	+6.3° (Ch)	113
D-Ribose diethyl mercaptal	(a) 83.5–84 (b) 82–83 (c) 83–84	(a) −25.6° (M); (b) −41.5° (W); (c) −25.9° (M)	(a) 114a (b) 245 (c) 114b

TABLE VII—*Continued*

Compound	M.p., °C.	$[\alpha]_D$ (solvent)	Reference
2,3,4,5-tetra-*O*-acetate	49.5–50	+25.1° (Ch); +27.7° (M)	238
2,3,4-tri-*O*-acetyl-5-*O*-benzoyl-	47–48	+14.5° (Ch)	245
2,3,4-tri-*O*-acetyl-5-*O*-benzyl-	liquid (b.p. 170–180° (b.T.)/10^{-4} mm.)	+9°	232
2,3,4-tri-*O*-acetyl-5-*O*-trityl-	96.5–97.5	−3.4° (Ch)	113
5-*O*-benzoyl-	100	0° (Ch)	245
2,3,4-tri-*O*-benzoyl-5-*O*-trityl-	147–148	+17.4° (Ch)	113
5-*O*-trityl-	—	−4.6° (Ch)	113
D-Ribose ethylene mercaptal	(a) 105–105.5° (b) 104–105 (c) 108	(a) −23.2° (M); (b) −23.1–23.9° (M); (c) −21° (Py); −25° (M); −29° (Et)	(a) 114a (b) 114b (c) 112
2,3,4-tri-*O*-acetyl-5-*O*-trityl-	127–128	−3.7° (Ch)	113
5-*O*-trityl-	—	−6.2° (Ch)	113
D-Ribose di-*N*-propyl mercaptal	83–83.5	−24.1 (M)	114a
D-Ribose diisopropyl mercaptal	(a) 97–97.5 (b) 96.5–97.5	(a) −48.6° (M); (b) −48.8° (M)	(a) 114a (b) 114b
2,3,4,5-tetra-*O*-acetate	102–103	+12.9° (Ch); +18.7° (M)	238
D-Ribose diisobutyl mercaptal	83.5–84	−18.6° (M)	114a
D-Ribose dibenzyl mercaptal	(a) 79.5–80 (b) 80	(a) −24.2° (M); (b) −19° (Et); −20° (M)	(a) 114a (b) 112
2,3,4-tri-*O*-acetyl-5-*O*-trityl-	109–110	−85.2° (Ch)	113
2,3,4,5-tetra-*O*-acetate (0.5 MeOH)	(a) (75 solvent free) 95 (b) 74–75	(a) −115° (Et); −97° (Ch); (b) −93.6° (Ch); −113° (M)	(a) 112 (b) 238
2,3,4-tri-*O*-benzoyl-5-*O*-trityl-	140–140.5	−70.2° (Ch)	113
5-*O*-trityl-	—	−82.5° (Ch)	113
mercuric chloride complex	183 (D)	—	112
D-Ribonic acid	112–113	−17.3° (M)	240
2,3,4,5-tetra-*O*-acetate	(a) 139–140 (b) 138–139 (c) 138–139	(a) −27.5° (5% HAc); (b) −24.4° (Ch); (c) —	(a) 240 (b) 255 (c) 169

phenylhydrazide	163	—	19
2,3,5-tri-O-methyl-, phenylhydrazide	108.5–109.5	—	134
D-Ribonamide	136–137	+16.5° (W)	255
2,3,4,5-tetra-O-acetyl-	(a) 123–124 (b) 123	(a) −35.5° (Ch); (b) —	(a) 255 (b) 169
2,3,4,5-tetra-O-propionyl-	123–124	—	169
D-Ribonolactone	(a) 77 (b) 80	(a) —; (b) —	(a) 70 (b) 343
tri-O-acetyl-	54–56	+27° (Ch)	240
2,3,4-tri-O-methyl-	liquid (b.p. 93–95/0.05 mm.)	+69.3° (Ch)	136
2,3,5-tri-O-methyl-	(a) 18.5–19 (b) —	(a) −20.2° (1) → −10.6° (E) (W); (b) +56.8° (Ch); −19° (W)	(a) 134 (b) 220
Methyl D-ribonate			
2,3,4,5-tetra-O-acetate	87–89	−13.0° (M)	255
D-Ribonyl chloride			
2,3,4,5-tetra-O-acetate	(a) 75 (b) 74–76	(a) —; (b) −43° (Ch)	(a) 169 (b) 255
D-Ribononitrile			
2,3,4,5-tetra-O-acetate	71–72	+34.45° (Ch)	240
Ethylthiol-D-ribonate			
2,3,4,5-tetra-O-acetate	87–87.5	+17° (Ch)	239
D-Ribose 5-phosphate	(a) — (b) — (c) —	(a) +16.54° (1N HCl); (b) +16.5° (N/6 HCl); (c) +17.2° (0.02N H_2SO_4	(a) 175 (b) 180 (c) 45
barium salt	(a) — (b) —	(a) +6.5° (W); (b) +5.99° (W)	(a) 45 (b) 175
DL-Ribose	83–84	—	100
L-Ribose	(a) 85–87 (b) 87 (c) 87 (d) —	(a) +22.6° (W); (b) +23.2° (after 2 min.) → +24.0 (after 28 hr.) (W); (c) +18.8° (W); (d) +14°	(a) 76 (b) 129 (c) 15 (d) 69
3,4-di-O-acetyl-, 1,2-ortho-3′-O-acetoxyacetonyl acetate	97–98	+11.8° (Ch)	102
1,2,3,4-tetra-O-acetyl-	109.5–110	+56° (Ch)	102
p-bromophenylhydrazone	(a) 165–165.5 (b) 170–172 (c) 164–165 (d) 165	(a) —; (b) −10.67° (Et); (c) —; (d) —	(a) 102 (b) 76 (c) 14 (d) 15

TABLE VII—*Concluded*

Compound	M.p., °C.	$[\alpha]_D$ (solvent)	Reference
L-Ribopyranosyl bromide			
2,3,4-tri-*O*-acetate	94.5–95.5	+224.8° (Ch)	102
L-Ribonic acid	104–105	+17.6° (after 4 min.) → −4.6° (after 30 days) (W)	344
phenylhydrazide	162–164	—	14
L-Ribonamide	(a) 137–138 (b) 136–137	(a) −16.4° (W); (b) −15.7° → −2.8° (E) (W)	(a) 345 (b) 346
L-Ribonolactone	(a) 79–80 (b) — (c) 72–76	(a) −17.8° (W); (b) −6.9°; (c) −18°	(a) 74 (b) 344 (c) 14
Ribitol	164–165	—	256
2,3,4,5-tetra-*O*-acetyl-	55–57	—	257
1-chloro-1-deoxy-2,3,4,5-tetra-*O*-acetyl-	134–135	—	257
1-nitro-1-deoxy-2,3,4,5-tetra-*O*-acetyl-	64–65	−7.8° (Ch)	5
di-*O*-benzylidene-	164–165	—	256
1-nitro-1-deoxy-3,5-*O*-benzylidene-	106–107	−38.8° (Ch) (for D-isomer)	5
5-phosphate (sodium salt)	—	−8.9° (W); −6.4° (50% saturated borax)	219

TABLE VIII

DERIVATIVES OF 2-DEOXYRIBOSE[a]

Substance	M.p., °C.	$[\alpha]_D$ (solvent)	Reference
2-Deoxy-D-ribose	(a) 78 (b) 91 (c) 87–90 (d) 96–98 (e) 80	(a) −90.6° (I) → −40° (Py); −60° (I) → −50° (W); (b) −87.8° (Py); (c) −55.2° (E) (W); (d) −91° → −58° (after 61.5 min.) (W); (e) −56.5° (W)	(a) 266 (b) 276 (c) 6 (d) 277 (e) 278, 279
1,3,4-tri-*O*-acetate	(a) Syrup (b.p. 180° (b.T.)/0.1 mm.) (b) 98	(a) 52.5° (Ch); (b) −171.8° (Ch)	(a) 302 (b) 252
1,3,4-tri-*O*-benzoate	127	−65° (Ch)	252
benzylphenylhydrazone	(a) 128 (b) 129 (c) 125.5–126.5	(a) −17.5° (Py); (b) −16.5° (Py); (c) −15.25°	(a) 265 (b) 278, 279 (c) 289
p-nitrophenylhydrazone	160	−11.1° (Et)	252
Methyl 2-deoxy-β-D-ribopyranoside	(a) 81–82 (b) liquid (b.p. 112° (b.T.)/0.02 mm.)	(a) +218.5° (W); (b) —	(a) 275 (b) 251
3,4-*O*-isopropylidene-	liquid (b.p. 100–115°/12 mm.)	−47.8° (W)	252
3,4-di-*O*-tosyl-	104–107	−115.5° (Ch)	252
Methyl 2-deoxy-α,β-D-ribofuranoside	liquid (b.p. 115–125° (b.T.)/0.45 mm.)	+38.4° (HAc)	281
3,5-di *O*-tosyl-	liquid	−121° (Ch)	252
N-Phenyl-2-deoxy-D-ribosylamine	(a) 165–166 (b) 175–176 (c) 168 (d) 172–173	(a) —; (b) +20.5° (E) (Et); (c) +19.5° (Et); (d) +17.4 (Et)	(a) 251 (b) 6 (c) 277 (d) 284
Adenine-9-(2′-deoxy-D-ribofuranoside)	(a) (S, 120.5) 181 (b) (S, 162.5) 187.8	(a) —; (b) −26.9° (W)	(a) 267 (b) 347
5-(?) *O*-phosphate	142.5	—	270

[a] See footnote *a*, Table VII, page 65.

TABLE VIII—*Continued*

Substance	M.p., °C.	$[\alpha]_D$ (solvent)	Reference
1-(2′-Deoxy-D-ribopyranosyl)-benzimidazole			
hydrochloride hydrate	150	−34.5° (W)	166
picrate	170	−14.8°	166
3′,4′-di-*O*-acetate picrate	167–168	−8.6° (Py)	166
1-(2′-Deoxy-D-ribopyranosyl)-5,6-dimethylbenzimidazole			
monohydrate	160	+30.9° (Py)	166
picrate	203	—	166
3′,4′-di-*O*-acetate	118	+2.6° (Ch)	166
3′,4′-di-*O*-acetate picrate	203	—	166
3-(2′-Deoxy-D-ribofuranosyl)-cytosine	(a) 193 (b) 214 (c) 206–208	(a) +40° (W); (b) +82.4° (1*N* NaOH); (c) —	(a) 264 (b) 268 (c) 306
picrate	darkens at 191° and then decomposes	—	306
5′(?)-*O*-phosphate	185–187	—	270
9-(2′-Deoxy-D-ribofuranosyl)-guanine	(a) without m.p. (b) without m.p. (c) —	(a) −37.5° (1*N* NaOH); (b) −47.7° (1*N* NaOH); (c) −36.0° (1*N* NaOH)	(a) 346 (b) 268 (c) 264
9-(2′-Deoxy-D-ribofuranosyl)-hypoxanthine	(a) (S, 202) —; (b) 219 (c) without m.p.	(a) −21° (aq. NaOH); (b) −22.9° (1*N* NaOH); (c) —	(a) 264 (b) 268 (c) 306
(2′-Deoxy-D-ribopyranosyl)theophylline (Form I)	212	−9° (W)	302
3′,4′-di-*O*-acetate	152	−63° (Ch)	302
(2′-Deoxy-D-ribopyranosyl)theophylline (Form II)	190	−21.5° (W)	302
3′,4′-di-*O*-acetate	207–208	+51° (Ch)	302
3-(2′-Deoxy-D-ribofuranosyl)-thymine	(a) 185 (b) 187 (c) 184.5–185.5	(a) +32.5° (1*N* NaOH); (b) +32.8° (1*N* NaOH); (c) —	(a) 264 (b) 268 (c) 306

(2′-Deoxy-D-ribosyl)uracil	163	+50 ± 2° (1*N* NaOH)	52
Calcium 2-deoxy-D-ribonate	—	−5.6° (W)	276
Barium 2-deoxy-D-ribonate	—	+0.17° (W)	276
Methyl 3,4,5-tri-*O*-methyl-2-deoxy-D-ribonate	102	—	276
2-Deoxy-D-ribonamide	137	—	276
2-Deoxy-D-ribonolactone	154–155	−2.5° (W)	276
2-Deoxy-D-ribonic acid phenylhydrazide	176–178	—	276
2-Deoxy-D-ribose-1-phosphate (cyclohexylamine salt)	(S and D at 152)	—	308
2-Deoxy-L-ribose	(a) *ca.* 90 (b) 80 (c) 92–95	(a) —; (b) +91.7° (I) → +40.5° (E) (Py); (c) + 80° (I) → +59° (after 41 min.) (W)	(a) 280 (b) 266 (c) 277
benzylphenylhydrazone	(a) 125–126 (b) 127–129	(a) +17.5° (Py); (b) —	(a) 266 (b) 280
dibenzyl mercaptal	66–70	−12.0° (Ch)	277
diethyl mercaptal	liquid (b.p. 170–175°/0.06 mm.)	+8.8° (Et)	277
3,4-di-*O*-methyl-	liquid (b.p. 90–100°/0.3 mm.)	+73.8° (W)	281
3,5-di-*O*-methyl-	liquid	−42.5° (W)	281
Methyl 2-deoxy-α-L-ribopyranoside	99–100	−43.4° (W); −70° (M); −176° (Ch)	281
Methyl 2-deoxy-β-L-ribopyranoside	83–84	+202.3° (W); +210° (M); +193° (Ch)	281
3,4-di-*O*-methyl-	liquid (b.p. 85–95° (b.T.)/12 mm.)	+224° (W)	281
Methyl 2-deoxy-α,β-L-ribofuranoside	liquid (b.p. 120–140° (b.T.)/0.14 mm.)	−27.6° (W)	281
3,5-di-*O*-methyl-	liquid (b.p. 96–101° (b.T.)/12–15 mm.)	+5.1° + 5.9° (W)	281
Ethyl 2-deoxy-L-riboside	liquid (b.p. 110–120°/0.1 mm. approx.)	−26.5° (Et)	277

TABLE VIII—*Concluded*

Substance	M.p., °C.	$[\alpha]_D$ (solvent)	Reference
N-Phenyl-2-deoxy-L-ribosylamine	(a) 169.5–170.5 (b) 172–173	(a) —; (b) −142° (after 10 min.) → −58° (after 240 min.) (Py); −6.6° (M)	(a) 277 (b) 160
2-Deoxy-L-ribonic acid	(a) — (b) —	(a) + 8.5° (I) → −10.7° (W); (b) +8.5° (W + HCl)	(a) 74 (b) 266
barium salt	(a) — (b) —	(a) −0.4° (W); (b) −0.43° (W)	(a) 74 (b) 266
phenylhydrazide	145–146	—	277
2-Deoxy-L-ribonolactone	(a) — (b) 153–155 (c) 153–155 (d) —	(a) −12.2° (W); (b) −2.1° (c) −2.73° (d) −13.8° (I) → −14.5° (after 8.29 days) (W)	(a) 266 (b) 348 (c) 276 (d) 277
3,4-di-*O*-methyl-	liquid (b.p. 106–112°/0.2 mm.)	+43.6° (I) → 0° (after 6 days) (0.123 *M* NaCl)	281
3,4-di-*O*-methyl-, phenylhydrazide	67–72	—	281
3,5-di-*O*-methyl-	liquid (b.p. 100–110° (b.T.)/0.3 mm.)	−33.3° (W)	281

TABLE IX

ADDENDUM TO TABLES OF CONSTANTS

Compound	M.p., °C.	$[\alpha]_D$	Ref.
β-D-Ribose			
1,2,3,5-tetra-*O*-acetyl	81–82	—	327
1-*O*-acetyl-2,3,5-tri-*O*-benzoyl	130–131	+44.2° (Ch)	338
1,2,3,5-tetra-*O*-benzoyl	121–122	+17.0° (Ch)	338
2,3,4-tri-*O*-benzoyl	136–137	−42.2° (Ch)	337
2,3,5-tri-*O*-benzoyl	111–112	+67.7° → +65.6° in 2 days (Ch)	338
Aldehydo D-ribose tetra-*O*-benzoate	112–113	−22° (Ch)	338
diisobutylthio-acetal	108–109	+8.5° (Ch)	338
2,4-dinitrophenylhydrazone	150 (resolidifies and then remelts at 183–185)	−69° (U.S.P. Ch)	338
Benzyl β-D-ribopyranoside	103–104	−109° (W)	337
2,3,4-tri-*O*-benzoate	144–145	−108° (Ch)	337
Benzyl β-D-ribofuranoside	95–96	−60.5° (W)	338
2,3,5-tri-*O*-benzoate	87–88	+14.9° (Ch)	338
Ribose-2-phosphate			
barium salt	—	−6.8° (W)	334
brucine salt	—	−27.5° (W:Py, 1:1)	334
sodium salt	—	−10.7° (W); −14.6° (W + borax)	334
Ribose-3-phosphate			
barium salt	—	−6.8° (W)	334
brucine salt	—	−35° (W:Py, 1:1)	334
sodium salt	—	−10.2° (W); −50° (W + borax)	334
5,6-Dimethylbenzimidazole-1-α-D-ribofuranoside	198	+14° (Py)	327
picrate	(a) 210; (b) 201–202	(a) +9° (Py); (b) +12 ± 2° (Py)	(a) 327 (b) 326

TABLE IX—*Concluded*

Compound	M.p., °C.	$[\alpha]_D$	Ref.
5,6-Dimethylbenzimidazole-1-β-D-ribofuranoside	197–200	−44° (Py)	327
picrate	175	—	327
9-β-D-Ribofuranosylpurine	181–182	−48.6 (W); −61° (0.1 *N* NaOH); −22° (0.1 *N* HCl)	317
6-chloro	170–171 (dec.) (dependant on rate of heating)	—	317
Ribitol			
penta-*O*-benzoate	108–109	—	338
2-Phosphate (barium salt)	—	+6.0° (W)	334
5,6-Dimethylbenzimidazole-1-(2′-deoxy-D-ribopyranoside)			
picrate	200–201 (dec.)	+27 ± 2° (Py)	326
D-Ribose			
1-*O*-acetyl-2,3,5-tri-*O*-benzoyl	129–130	—	340
1,2-di-*O*-acetyl-3,5-di-*O*-benzoyl	127–128	−3° (Ch)	340
3,5-Di-*O*-benzoyl-D-ribofuranosyl bromide	104–105 (dec.)	+96° (3 min.) → +27° (60 min.) (Ch)	340
3,5-Di-*O*-benzoyl-1,2-*O*-(1,hydroxy-benzylidene)-α-D-ribose	142–143	+86° (Ch)	340
3,5-Di-*O*-benzoyl-1,2-*O*-(1-hydroxy-ethylidene)-α-D-ribose	129–130	+66.4° (Ch)	340
3-Carbamyl-1-D-ribosylpyridinium bromide	147 (dec.)	—	342
monoacetyl	170 (dec.)	—	342
triacetyl	142	—	342

CHAPTER 3

Chemistry of Purines and Pyrimidines

AARON BENDICH

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I. Introduction

1. Historical

Pyrimidines and purines are intimately associated with all the living systems which have thus far been studied, and have been found, as Rivers put it,[1] even ". . . in the twilight zone between the living and the non-living," the viruses. These compounds, in the free form as well as i wide variety of chemical combinations, have caught the attention imagination of biologists, enzymologists, chemists, oncologists, physic and many students of chemotherapy.

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[1] T. M. Rivers, "Viral and Rickettsial Infections of Man," 2nd ed., p. 5. Lippincott, Philadelphia, 1952.

Recognition of this group of compounds dates back to the discovery 1776 of uric acid by Scheele and by Bergmann and of alloxan in 1818 Brugnatelli.[2] It includes the pioneering studies on uric acid of Liebig Wöhler[3,4] begun in 1834 and those of Baeyer[5] in 1863 which led to cyclic formulation (1875) of uric acid and guanine by Medicus[6] and pyrimidine nucleus (1884–5) by Pinner.[7,8] The classic studies on puri (1882–1907) by Fischer have been collected in a single volume[9] which contains a description of the early work. There are other excellent treat which deal with the history and development of these substances and th occurrence as components of the nucleic acids.[10-14]

2. Nomenclature

The term pyrimidine was coined by Pinner[8] from a combination of words pyridine and amidine. He was the first[8] to point out the structu similarity of pyrimidines to benzene, pyridine, and *s*-triazine, and, cordingly, depicted the ring system in the form of a regular hexagon.[7 Although the hexagonal representation of pyrimidines (I) has been adopt by *Chemical Abstracts*[16] and *The Ring Index*,[17] many authorities have p ferred the rectangular form (II)[9,13,14,18,19] numbered as shown (II), or n III.[20] Both systems, I and II, have been used in a single work.[21] Confusion also arises from nomenclature inconsistency. For example, in *Chemical Abstracts*, IV is called 2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione, whereas V is called 6-methyl-2,4-pyrimidinediol,[16] and isobarbituric acid is listed as 5-hydroxy-2,4(1*H*,3*H*)-pyrimidinedione.[22]

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[2] G. Brugnatelli, *Ann. Chem. Phys.* [2] **8,** 201 (1818).

[3] J. Liebig, *Ann.* **10,** 47 (1834).

[4] F. Wöhler and J. Liebig, *Ann.* **26,** 241 (1838).

[5] A. Baeyer, *Ann.* **130,** 129; **131,** 291 (1864); See also *Ann.* **127,** 199 (1863).

[6] L. Medicus, *Ann.* **175,** 230 (1875).

[7] A. Pinner, *Ber.* **17,** 2519 (1884).

[8] A. Pinner, *Ber.* **18,** 759, 2845 (1885).

[9] E. Fischer, "Untersuchungen in der Puringruppe." Springer, Berlin, 1907.

[10] C. Brahm and J. Schmid, *in* "Biochemisches Handlexikon" (Abderhalden, ed Vol. 4, pp. 1014, 1131. Springer, Berlin, 1911.

[11] W. Jones, "Nucleic Acids, Their Chemical Properties and Physiological Conduc Longmans, Green and Co., London, 1920.

[12] R. Feulgen, "Chemie und Physiologie der Nucleinstoffe nebst Einführung in Chemie der Purinkorper." Borntraeger, Berlin, 1923.

[13] P. A. Levene and L. W. Bass, "Nucleic Acids." Chemical Catalog Co., New Yo 1931.

[14] T. B. Johnson and D. A. Hahn, *Chem. Revs.* **13,** 193 (1933).

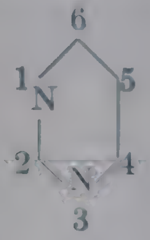
[15] A. Pinner, *Ber.* **20,** 2361 (1887); **22,** 1600, 1612 (1889); **26,** 2122 (1893).

[16] *Chem. Abstr.* **39,** 5867 (1945).

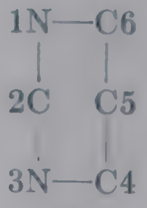
[17] A. M. Patterson and L. T. Capell, "The Ring Index," p. 52. Reinhold, New Yo 1940.

[18] T. B. Johnson, *in* "Organic Chemistry, An Advanced Treatise" (Gilman, ed Vol. 2, p. 948. Wiley, New York, 1938.

[19] F. Schlenk, *Advances in Enzymol.* **9,** 455 (1949).



I



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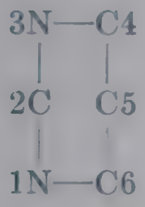

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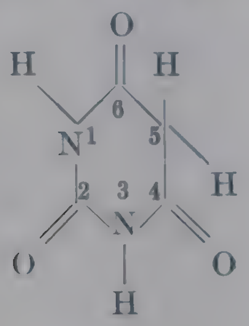
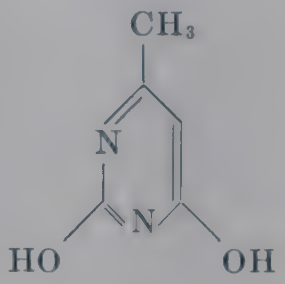


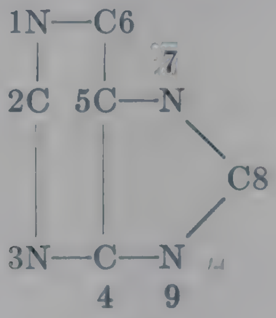
IV



V

Since the pyrimidine nucleus is symmetrical about a plane including C-2 and C-5, alternative numbering is possible (see II and III above).

Fischer[9,23] introduced the term purine which he derived from *purum* and *uricum*, and employed the widely used construction (VI) based upon the Medicus formula now ascribed to uric acid. This heterocycle (VI) consists of a fused pyrimidine and imidazole nucleus in which carbon atoms 4 and 5 are shared by both rings.



VI

The ring and numbering system employed by *Chemical Abstracts*[16] and

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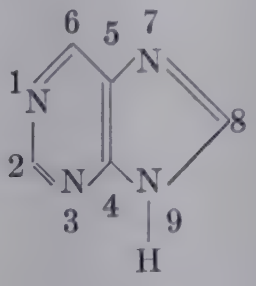
[20] V. Meyer and P. Jacobson, "Lehrbuch der organischen Chemie." Vol. II, p. 1172. De Gruyter, Berlin and Leipzig, 1920.

[21] A. A. Morton, "The Chemistry of Heterocyclic Compounds," p. 483. McGraw-Hill, New York, 1946.

[22] *Chem. Abstr.* **44,** 12451, 12845 (1950).

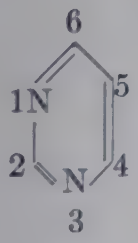
[23] E. Fischer, *Ber.* **31,** 2550 (1898); **32,** 435 (1899).

The Ring Index[17] for purines is also used in this text (VII), and, for c sistency and to indicate more clearly the interrelationships between puri and pyrimidines, the style (VIII) for pyrimidines is used here. The h



VII

Purine



VIII

Pyrimidine

agonal representations (VII and VIII) are preferred since they confor closest to those revealed by X-ray studies.[24]

The actual structures of pyrimidines and purines depend upon ma conditions (see below) and, even when the effect of a condition (such pH) is understood, it is not always possible to write a single structure f a particular compound. For the sake of brevity it is necessary, therefor to be arbitrary in representing the structures. Except where otherwi noted, amino, hydroxy, mercapto, or other derivatives of these heter cycles will be written as if they had been formed by a replacement of hydrogen atom, leaving the Kékulé-type ring intact. Thus uric acid referred to as 2,6,8-trihydroxypurine and 5-methylcytosine as 2-hydrox 5-methyl-6-aminopyrimidine, without regard to the obvious operation lactim-lactam or amino-imino tautomerism. It is to be understood th such representations do not necessarily reflect an actual state of a molecul

3. Occurrence and Distribution

a. Purines and Pyrimidines of Nucleic Acid Origin

Thus far, only two purines are recognized as universal and normal co stituents of nucleic acids (PNA and DNA). Adenine[25] was discovered a isolated from an acid hydrolysate of "nuclein" of beef pancreas in 1885 b Kossel.[26,27] He succeeded[28] in converting adenine into hypoxanthine (6-h droxypurine) upon reaction with nitrous acid. Although Medicus[6] propose the correct cyclic structure for hypoxanthine, it was not until 1897 that concrete chemical evidence for the structure of adenine (IX) was made available by Fischer in his partial synthesis of both adenine and hypoxanthine from 2,6,8-trichloropurine[29] obtained from uric acid:

---

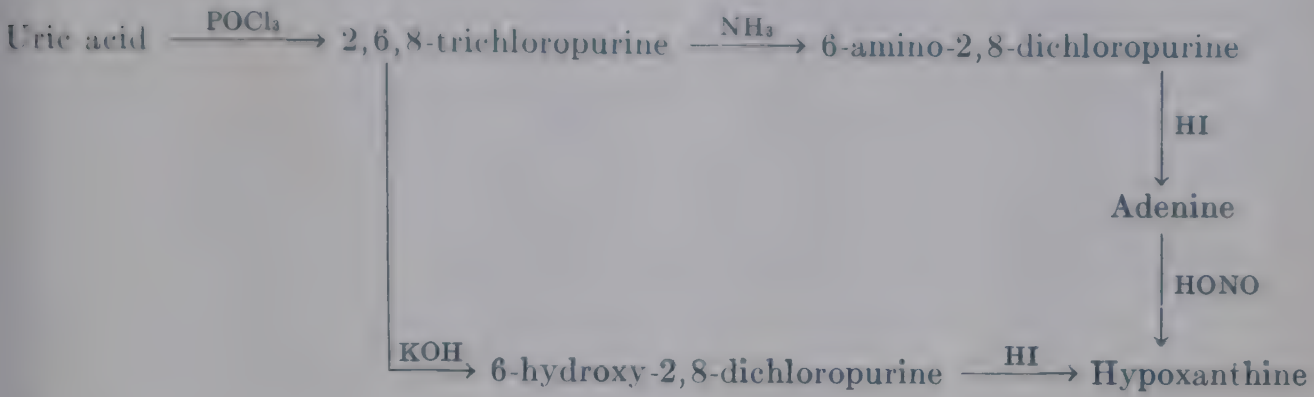
[24] D. O. Jordan, *Progress in Biophysics* **2,** 51 (1951); *Ann. Rev. Biochem.* **21,** 209 (195

[25] Beilstein's Handbuch der organischen Chemie.," 4th ed., **26,** 420 (1937). (Herei after referred to as "Beilstein.")

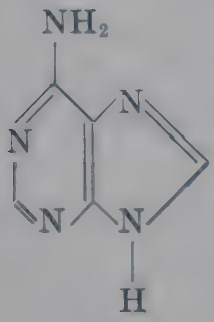
[26] A. Kossel, *Ber.* **18,** 79 (1885).

[27] A. Kossel, *Z. physiol. Chem.* **10,** 248 (1886).

[28] A. Kossel, *Ber.* **18,** 1928 (1885).

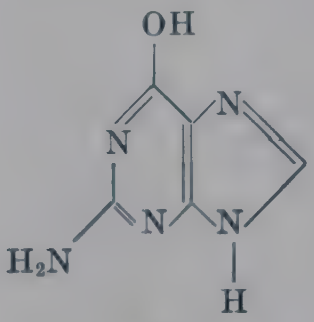


Total syntheses of adenine and hypoxanthine were accomplished in 1904 by Traube.[30] (The Traube syntheses are discussed on page 131.) Certain of the chemical and physical properties of adenine and other purines and pyrimidines are listed in Table I and are discussed below.



IX

Adenine



X

Guanine

Guanine (2-amino-6-hydroxypurine, X)[31] was found in 1844 in the excreta (*guano*) of birds[32,33] before it was recognized as a nucleic acid constituent.[34] The accepted cyclic structure for guanine, postulated by Medicus,[6] received considerable support from its conversion by nitrous acid to xanthine (2,6-dihydroxypurine) and the chloric acid oxidation of guanine to guanidine and parabanic acid by Strecker.[35] The relationship of guanine to the purine system (uric acid) was elucidated by Fischer.[29] These rea tions are summarized below:

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[29] E. Fischer, *Ber.* **30,** 2226 (1897).

[30] W. Traube, *Ann.* **331,** 64 (1904).

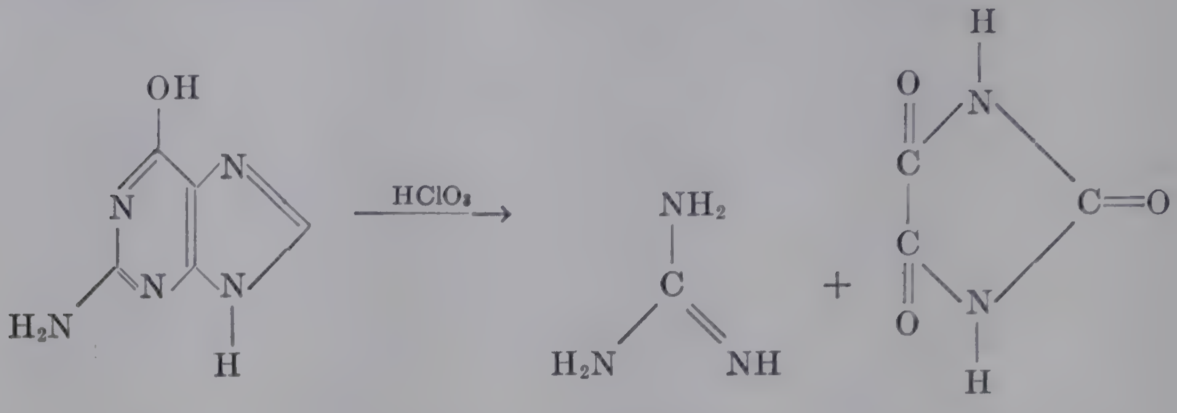
[31] Beilstein, **26,** 449 (1937).

[32] Magnus, *Ann.* **51,** 395 (1844).

[33] B. Unger, *Ann.* **58,** 18 (1846); **59,** 58 (1846).

[34] A. Kossel, *Z. physiol. Chem.* **8,** 404 (1883–1884); see also *Z. physiol. Chem.* **3,** 284 (1879); **5,** 152, 267 (1881).

[35] A. Strecker, *Ann.* **108,** 129, 141 (1858); **118,** 151 (1861).



X Guanidine Parabanic acid

$$\begin{tikzcd}
\text{2,6,8-trichloropurine} \arrow[r, "KOH"] \arrow[d, "C_2H_5ONa"] & \text{6-hydroxy-2,8-dichloropurine} \arrow[r, "NH_3"] & \text{2-amino-6-hydroxy-8-chloropurine} \arrow[d, "HI"] \\
\text{2,6-diethoxy-8-chloropurine} \arrow[r, "HI"] & \text{Xanthine} & \text{Guanine} \arrow[l, "HONO"']
\end{tikzcd}$$

Total syntheses of guanine and xanthine were first described by Traube.

Five pyrimidines have been isolated to date from nucleic acid source The first to be discovered, thymine (2,6-dihydroxy-5-methylpyrimidine 5-methyluracil, XI),[37] was isolated in 1893 by Kossel and Neumann[38] from acid hydrolysates of the nucleic acids of calf thymus and beef splee These investigators, as well as Jones[40] and Steudel and Kossel,[41] found tha thymine was not identical with the isomeric 2,6-dihydroxy-4-methylpyrimidine which had been synthesized and named 4-methyluracil by Behrend in 1884. The studies of Steudel,[43] in which urea was obtained followin permanganate oxidation of thymine, led him to conclude that XI expresse the proper structure. This conclusion was soon confirmed by a number

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[36] W. Traube, *Ber.* **33,** 1371, 3035 (1900).

[37] Beilstein, **24,** 353 (1936).

[38] A. Kossel and A. Neumann, *Ber.* **26,** 2753 (1893).

[39] A. Kossel and A. Neumann, *Ber.* **27,** 2215 (1894).

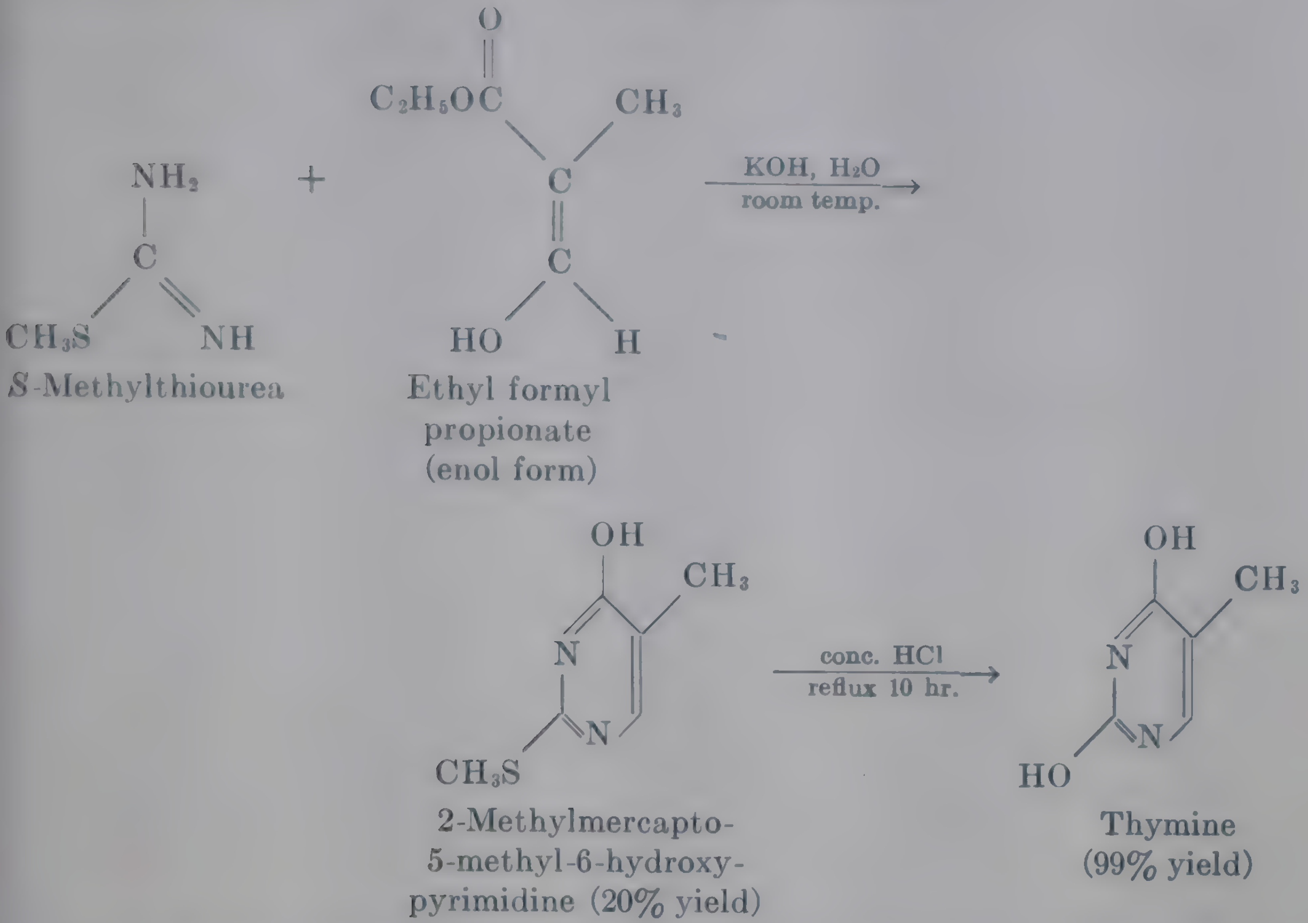
[40] W. Jones, *Z. physiol. Chem.* **29,** 20 (1899).

[41] H. Steudel and A. Kossel, *Z. physiol. Chem.* **29,** 303 (1899).

[42] R. Behrend, *Ann.* **229,** 1 (1885).

[43] H. Steudel, *Z. physiol. Chem.* **30,** 539 (1900).

syntheses of thymine,[44-47] one of which[45] is illustrated:


S-Methylthiourea
Ethyl formyl propionate (enol form)
KOH, H2O
room temp.
2-Methylmercapto-5-methyl-6-hydroxy-pyrimidine (20% yield)
conc. HCl
reflux 10 hr.
Thymine (99% yield)

Thymine, a ubiquitous component of the deoxy type of nucleic acid (see Chapters 4 and 10), has not yet been found in the pentose nucleic acids or in the free state in natural sources, although it has been isolated as an *N*-xyloside (spongothymidine) from sponges (see below).

In 1894, Kossel and Neumann also[39] discovered as a cleavage product of calf thymus nucleic acid a basic substance which they named cytosine (2-hydroxy-6-aminopyrimidine, XII).[48] Certain of its salts were described[39, 49-51] and this led to its correct empirical composition. Since uracil was obtained from nitrous acid deamination, and biuret (rather than guanidine) from permanganate oxidation, Kossel and Steudel[51] proposed the accepted structure of cytosine. The synthesis of cytosine[52] furnished further proof of its structure. The synthesis is analogous to that for thymine previously described above in that *S*-ethylthiourea and ethyl formylacetate were

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[44] H. Steudel, *Z. physiol. Chem.* **32,** 241 (1901).

[45] H. L. Wheeler and H. F. Merriam, *Am. Chem. J.* **29,** 478 (1903).

[46] O. Gerngross, *Ber.* **38,** 3408 (1905).

[47] H. L. Wheeler and D. F. McFarland, *Am. Chem. J.* **43,** 19 (1910).

[48] Beilstein, **24,** 314 (1936).

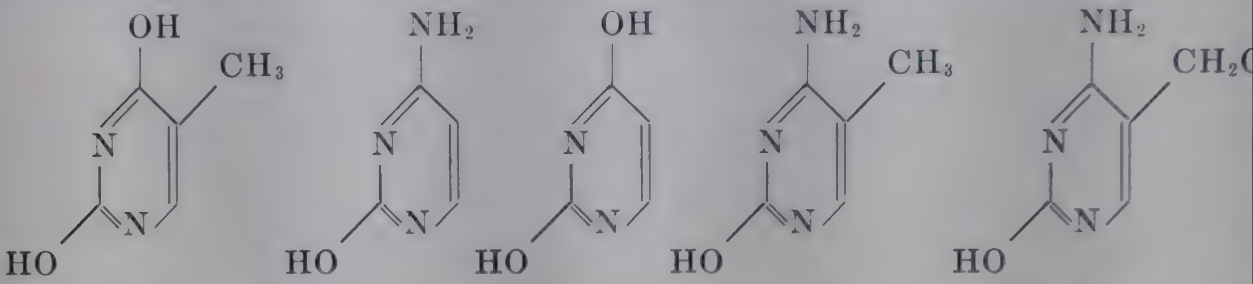
[49] A. Kossel and H. Steudel, *Z. physiol. Chem.* **37,** 177, 377 (1902–03).

[50] P. A. Levene, *Z. physiol. Chem.* **38,** 80 (1903).

[51] A. Kossel and H. Steudel, *Z. physiol. Chem.* **38,** 49 (1903).

[52] W. L. Wheeler and T. B. Johnson, *Am. Chem. J.* **29,** 492 (1903).

condensed in alkaline solution at room temperature to afford 2-ethylm capto-6-hydroxypyrimidine. The hydroxyl group was replaced (phospho pentachloride) by chlorine, which in turn was replaced by an amino gro upon treatment with alcoholic ammonia. The ethylmercapto group w replaced by hydroxyl upon hydrolysis (125°) with hydrobromic acid. was found[53] that the synthetic cytosine and that obtained from wh germ and spleen nucleic acids were identical. With the exception, recen reported, of the DNA of T- even-numbered bacteriophages of *E. coli*,[54] specimens of PNA and DNA in which the pyrimidines have been char terized have been found to contain cytosine.



XI Thymine

XII Cytosine

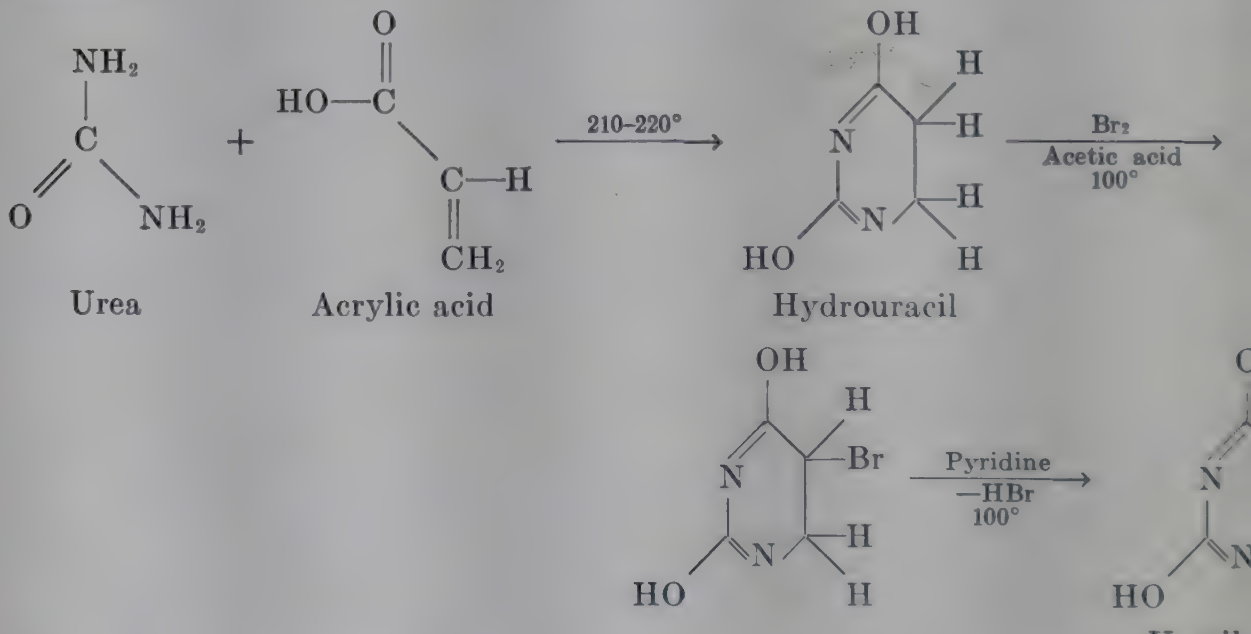
XIII Uracil

XIV 5-Methylcytosine

XV 5-Hydroxymethyl cytosine

Pyrimidines occurring in the nucleic acids

Following a procedure for the isolation of thymine from herring sperm Ascoli[56] isolated from yeast nucleic acid (1900–1901) a new compou ($C_4H_4N_2O_2$) which, as he pointed out, corresponded in empirical compo tion to the previously postulated (cf. Behrend[42]) *uracil* (XIII).[57] This su mise was confirmed by the following synthesis of uracil by Fischer a Roeder,[58] who considered their product to be identical with the natu substance:



Urea

Acrylic acid

Hydrouracil

Uracil

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[53] W. L. Wheeler and T. B. Johnson, *Am. Chem. J.* **29**, 505 (1903).

Uracil was also isolated from the nucleic acids of wheat germ ("Triticonuclein"[59]), calf thymus and herring sperm,[60] and beef spleen,[61] although it was suspected[60,61] that the uracil might have arisen from cytosine[51,52,62] as a result of hydrolysis (10 % H_2SO_4 at 150°). Uracil is now recognized as a universal constituent of the nucleic acids of only the pentose (or ribose) type (Chapters 4 and 11). Uracil occurs naturally in the free form in ergot[63] and in more complex combinations (see below).

Until 1925, the composition of the nitrogenous constituents of the nucleic acids was described only in terms of the five pyrimidines and purines discussed above; in that year, Johnson and Coghill reported[64] the occurrence of a homologue of cytosine, *5-methylcytosine* (XIV),[65] among the hydrolytic products of a nucleic acid from tubercle bacilli, tuberculinic acid. (Johnson had been seeking this substance in natural sources for some twenty-one years.) It was concluded[64] that the picrate of the isolated product was crystallographically identical with that of synthetic 5-methylcytosine,[66] but no melting point was recorded for comparison. In 1949, an examination of "5-methylcytosine" prepared by Johnson revealed[67] it to be a mixture consisting mainly of cytosine. Although a careful chromatographic study[67] of the DNA from avian tubercle bacilli did not confirm the early report,[64] a paper chromatogram of an hydrolysate of DNA from calf thymus revealed the presence of a small component ("epicytosine"), the properties of which led Hotchkiss[68] to the tentative view that it might be 5-methylcytosine. Wyatt[69, 70] has found 5-methylcytosine as a definite, though minor, constituent of the DNA's of mammalian, fish, and insect sources, and as a major pyrimidine component of wheat germ DNA. The latter finding has been confirmed.[71] It could not be detected in bacterial[70]

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[54] G. R. Wyatt and S. S. Cohen, *Nature* **170,** 1072 (1952).

[55] W. Jones, *Z. physiol. Chem.* **29,** 461 (1900).

[56] A. Ascoli, *Z. physiol. Chem.* **31,** 161 (1900–01).

[57] Beilstein, **24,** 312 (1936).

[58] E. Fischer and G. Roeder, *Ber.* **34,** 3751 (1901).

[59] T. B. Osborne and I. F. Harris, *Z. physiol. Chem.* **36,** 85 (1902).

[60] A. Kossel and H. Steudel, *Z. physiol. Chem.* **37,** 245 (1902–03).

[61] P. A. Levene, *Z. physiol. Chem.* **38,** 80 (1903).

[62] W. Jones and M. E. Perkins, *J. Biol. Chem.* **62,** 557 (1924–25).

[63] R. Engeland and F. Kutscher, *Zentr. Physiol.* **24,** 589 (1910).

[64] T. B. Johnson and R. D. Coghill, *J. Am. Chem. Soc.* **47,** 2838 (1925).

[65] Beilstein, **24,** 355 (1936).

[66] H. L. Wheeler and T. B. Johnson, *Am. Chem. J.* **31,** 591 (1904).

[67] E. Vischer, S. Zamenhof, and E. Chargaff, *J. Biol. Chem.* **177,** 429 (1949).

[68] R. D. Hotchkiss, *J. Biol. Chem.* **175,** 315 (1948).

[69] G. R. Wyatt, *Nature* **166,** 237 (1950).

[70] G. R. Wyatt, *Biochem. J.* **48,** 581, 584 (1951).

[71] G. Brawerman and E. Chargaff, *J. Am. Chem. Soc.* **73,** 4052 (1951).

(including tubercle bacilli) or viral sources,[72] and has not been found in
pentosenucleic acids. A nucleotide[73] and nucleoside[74] of 5-methylcytos
have been isolated from the DNA of thymus and wheat germ, respective
An excellent discussion concerning this pyrimidine is available.[75]

Quite recently, the absence of cytosine in the DNA of coliphage T_2 v
reported.[76] Since hydrolysis of the DNA with 70% $HClO_4$ (100°), wh
was employed,[77] does not lead to a destruction of cytosine (see also Wyatt
and since other analyses[78,79] indicated its apparent presence in T_2 and
a reinvestigation was made by Wyatt and Cohen.[54] Of great interest
their discovery of the occurrence in the DNA's of T-even bacteriopha
(but not in the DNA's of thymus or of the host *E. coli*) of a new pyrimid
base, *5-hydroxymethylcytosine* (XV) in place of cytosine. The base X
which withstands the hydrolysis with 88% formic acid (175° for 30 mi
necessary for its liberation from the DNA's, but which is largely destroy
by the treatment with $HClO_4$, has spectral and chromatographic prop
ties (R_f value in isopropanol-water-HCl)[70] very similar to those of cytos
(and 5-methylcytosine). These considerations serve to explain the abo
mentioned apparent discrepancies.[76,78,79] A synthesis of the new base (
published), its nitrous acid deamination to a uracil-like derivative, a
the isolation of a nucleotide have been reported.[54,80]

Because of the importance of this new base (XV) in DNA, and its str
tural similarity to vitamins B_1 (thiamine, XVI) and B_6 (pyridoxine, XV
a more detailed discussion of the chemistry of hydroxymethylpyrimidi
and related compounds is given.

In an attempt to prepare *N*-methyluracil derivatives, Kircher[81] condensed 4-meth
uracil (XVIII)[42] with formaldehyde in either dilute acid or alkaline aqueous solut
and obtained 4-methyl-5-hydroxymethyluracil (XIX) in high yield. Proof of its str
ture was readily obtained upon its reduction to the known 4,5-dimethyluracil (XX).[4
The compound XIX was unexpectedly labile to hydrolysis, undergoing a carbon-carb
cleavage upon mere boiling with water, and the original 4-methyluracil (XVIII) a
formaldehyde were regenerated by this mild treatment. Other, ill-defined, produ
($C_{11}H_{12}N_4O_4$ and $C_{12}H_{14}N_4O_5$) resulted when XIX was subjected to hot mineral a
(cf. Endicott and Johnson[83, 84] for a description of similar substances made by treat

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[72] G. R. Wyatt, *J. Gen. Physiol.* **36,** 201 (1952).

[73] W. E. Cohn, *J. Am. Chem. Soc.* **72,** 2811 (1950); **73,** 1539 (1951).

[74] C. A. Dekker and D. T. Elmore, *J. Chem. Soc.* **1951,** 2864.

[75] G. R. Wyatt, *Exptl. Cell Research* **3,** Suppl. 2, 201 (1952).

[76] A. Marshak, *Proc. Natl. Acad. Sci. U. S.* **37,** 299 (1951).

[77] A. Marshak and H. J. Vogel, *J. Biol. Chem.* **189,** 597 (1951).

[78] J. D. Smith and G. R. Wyatt, *Biochem. J.* **49,** 144 (1951).

[79] L. L. Weed and S. S. Cohen, *J. Biol. Chem.* **192,** 693 (1951).

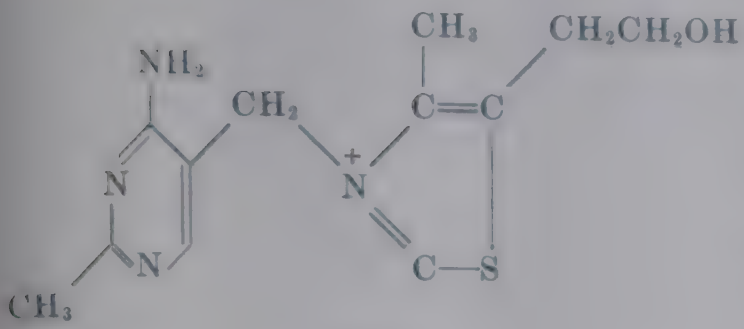
[80] G. R. Wyatt and S. S. Cohen, *Ann. inst. Pasteur* **84,** 143 (1953).

[81] W. Kircher, *Ann.* **385,** 293 (1911).

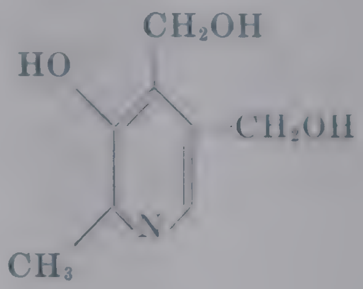
[82] J. Schlenker, *Ber.* **34,** 2812 (1901).

[83] M. M. Endicott and T. B. Johnson, *J. Am. Chem. Soc.* **63,** 1286 (1941).

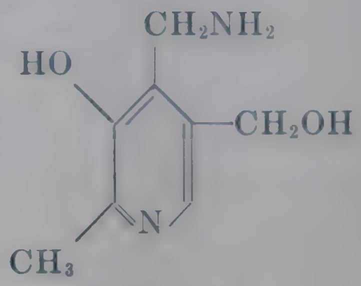
[84] M. M. Endicott and T. B. Johnson, *J. Am. Chem. Soc.* **63,** 2063 (1941).



XVI
Thiamine

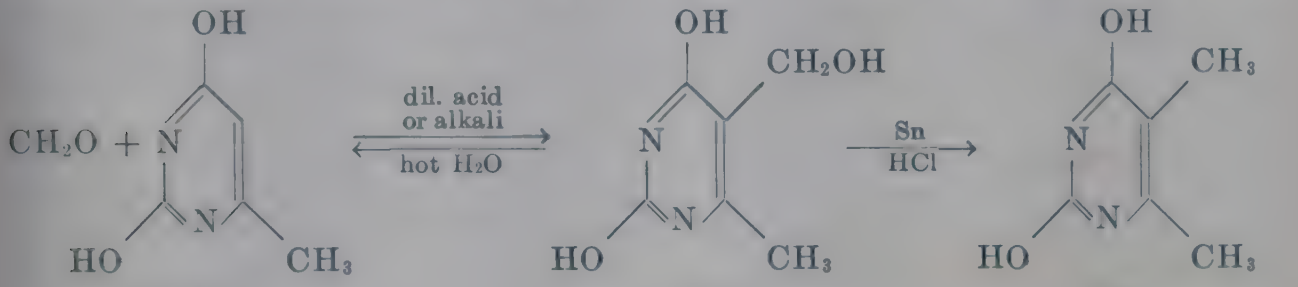


XVII
Pyridoxine

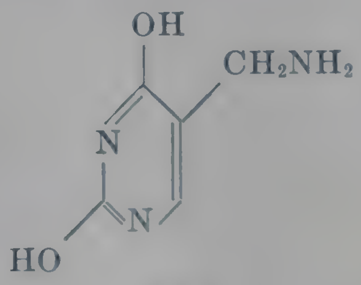


XVIIa
Pyridoxamine

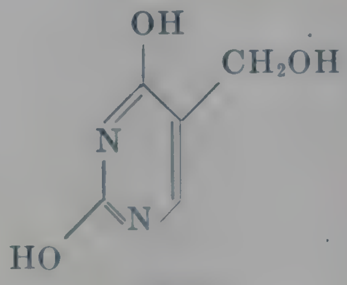
XVIII with chloromethyl ether). This instability of a pyrimidine-carbinol linkage has been found to be peculiar only to the hydroxymethyl at position 5, since such a group at position 4 (or 6) or 2, as in 4-hydroxymethylthymine[85, 86] and 4-hydroxymethyluracil[87] (originally considered as the simplest "nucleosides" of thymine and uracil, respectively), 2-methyl-4-hydroxy-6-hydroxymethylpyrimidine[88] and 2,6-bis(hydroxymethyl)-4-hydroxy-5-methylpyrimidine,[89] is not split off by hot water or hot mineral



XVIII XIX XX



XXI



XXII

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[85] T. B. Johnson and L. H. Chernoff, *J. Biol. Chem.* **14,** 307 (1913).

[86] T. B. Johnson and L. H. Chernoff, *J. Am. Chem. Soc.* **35,** 585 (1913).

[87] T. B. Johnson and L. H. Chernoff, *J. Am. Chem. Soc.* **36,** 1742 (1914).

[88] G. E. McCasland, D. S. Tarbell, R. B. Carlin, and N. Shakespeare, *J. Am. Chem. Soc.* **68,** 2390 (1946).

[89] G. E. McCasland and D. S. Tarbell, *J. Am. Chem. Soc.* **68,** 2393 (1946).

acid. Since no direct method has been developed for the preparation of substitutio derivatives (—OH, —NH$_2$, etc.) of the 5-methyl group of pyrimidines,[90] recourse ha been made to indirect methods[83-90] such as the Curtius technique. When this reactio was applied to ethyl-2-ethylmercapto-6-hydroxypyrimidine-5-acetate, the 5-carbeth oxymethyl group was converted to the ethyl ester of the methylurethan (—CH COOC$_2$H$_5$ → —CH$_2$NHCOOC$_2$H$_5$) and the urethan was hydrolyzed with liberation o ethyl mercaptan to yield 5-aminomethyluracil (or "thyminylamine," XXI).[91-93] Thi compound (XXI) was converted to 5-hydroxymethyluracil (or "thyminyl alcohol, XXII) by hydrolysis or nitrous acid deamination. ("Thyminyl alcohol," which shoul prove to be identical with the deamination product of 5-hydroxymethylcytosine o Wyatt and Cohen,[54] could not be prepared[93] by the action of formaldehyde upon uracil. Both the 5-hydroxymethyl- and the 5-aminomethyluracils were unstable to treatmen with hot water, and in the case of the latter compound (XXI) a carbon-carbon cleavag resulted with the formation of uracil, ammonia, and formaldehyde. The extreme in stability of these substances explains the difficulty[93] in preparing "absolutely pure' specimens. A similar instability towards hydrolysis of substituted 5-aminomethylura cils is manifested by the cleavage of these compounds to uracil, formaldehyde, and a substituted ammonia.[90] The chloromethylation[94] of 1,4-dimethyluracil with formaldehyde and HCl gives rise to 1,4-dimethyl-5-chloromethyluracil which is also unstabl towards hot water and (presumably via a conversion to 5-hydroxymethyl) decompose into formaldehyde and an insoluble dimethyluracil condensation product.[95] A simila behavior is described[83, 84] for 4-methyl-5-chloromethyluracil from which the unstabl 5-hydroxymethylpyrimidine (XIX) has been prepared.[84]

It will be pointed out in greater detail later that many analogies exist in the chemistry of pyrimidines and pyridines. For example, the 3 and 5 (or β) positions of pyridine and the chemistry of certain β-substituted pyridines are often akin to those of carbon 5 of pyrimidines. However, the hydroxy or aminomethyl groups of pyridoxine (XVII) and pyridoxamine (XVIIa) survive severe hydrolytic conditions with strong mineral acid.[96-98]

As to the stability of thiamine (XVI), most of the available data generally deal with the integrity of the intact vitamin, rather than the stability of the carbon-carbon linkage between C-5 of the pyrimidine and its side chain, but it might be mentioned that thiamine can be heated to 120°C at pH 3.5 without any decomposition.[99] The reader is referred to excellent discussions of the chemistry of the vitamin.[100-102] As for compounds related to the pyrimidine moiety of thiamine, certain of the derivatives possessing a substituent such as $—CH_3$, $—Cl$, or $—H$ at carbon 2 which is not capable of protopic change (a change such as

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[90] D. Riehl and T. B. Johnson, *Rec. trav. chim.* **59,** 87 (1940).

[91] T. B. Johnson and A. Litzinger, *J. Am. Chem. Soc.* **57,** 1139 (1935).

[92] A. Litzinger and T. B. Johnson, *J. Am. Chem. Soc.* **58,** 1936 (1936).

[93] T. B. Johnson and A. Litzinger, *J. Am. Chem. Soc.* **58,** 1940 (1936).

[94] R. C. Fuson and C. H. McKeever, *in* "Organic Reactions" (Adams, ed.), Vol. 1, p. 63. Wiley, New York, 1942.

[95] K. Schmedes, *Ann.* **441,** 192 (1925).

[96] M. Hochberg, D. Melnick, and B. L. Oser, *J. Biol. Chem.* **155,** 129 (1944).

[97] E. Cunningham and E. E. Snell, *J. Biol. Chem.* **158,** 491 (1945).

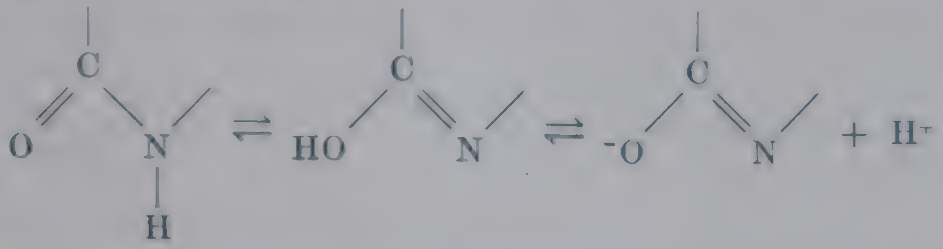
[98] D. Melnick, M. Hochberg, H. W. Himes, and B. L. Oser, *J. Biol. Chem.* **160,** 1 (1945).

[99] Ref. 101, p. 104.

[100] R. R. Williams and T. D. Spies, "Vitamin B$_1$ (Thiamin), and Its Use in Medicine." Macmillan, New York, 1938.

[101] H. R. Rosenberg, "Chemistry and Physiology of the Vitamins." Interscience, New York, 1942.

[102] F. A. Robinson, "The Vitamin B Complex." Wiley, New York, 1951.



etc.) do not show the instability associated with the corresponding uracil or cytosine derivatives described above. For example, a quantitative yield of 2-methyl-5-aminomethyl-6(4)-hydroxypyrimidine is obtained when the 5-urethanomethylpyrimidine is heated at 100°C. for 2 hours with concentrated HCl;[103] the aminomethyl group is converted to hydroxymethyl upon nitrous acid treatment for 7 hours at 100°C.[92] The hydroxymethyl group of 4-methyl-5-hydroxymethyl-6-aminopyrimidine persists after heating its 2-chloro derivative with zinc in water at 100° for 7 hours (no yields are cited, however).[104] Also, 2-methyl-5-bromomethyl-6-aminopyrimidine, in acid or neutral solution, withstands several treatments in the autoclave[105] (cf. Endicott and Johnson[84] and Schmedes[95]).

The simple replacement of a hydrogen atom of the stably-bound methyl group of 5-methyl-uracil or -cytosine by hydroxyl or amino (or chloro) gives rise to compounds possessing a carbon-carbon linkage which is unstable towards hot water or mineral acid, but not (in the case of 5-hydroxymethylcytosine (XV)) towards hot formic acid (which is a reducing agent). As yet, no theoretical explanation is available for this phenomenon and any explanation that is put forward must take into account the stability of the other derivatives mentioned above.

b. *Purines and Pyrimidines in Other Biological Sources*

A detailed catalog listing the widespread natural distribution of purines and pyrimidines has been compiled.[106] For other listings, the general references at the end of this chapter may be consulted. Because of this excellent reference literature, greater emphasis will be given to more recent developments.

Purine ($C_5H_4N_4$), the parent member of the series,[107] has recently been isolated as a 9-D-riboside ("Nebularine") from a mushroom.[108-110] Nebularine, the detailed structure of which has been established by synthesis as

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[103] A. R. Todd, F. Bergel, H. L. Fraenkel-Conrat, and A. Jacob, *J. Chem. Soc.* **1936,** 1601.

[104] H. Andersag and K. Westphal, *Ber.* **70,** 2035 (1937).

[105] W. J. Robbins and F. Kavanagh, *Proc. Natl. Acad. Sci. U. S.* **24,** 141 (1938).

[106] C. Wehmer and M. Hadders, *in* "Handbuch der Pflanzenanalyse" (G. Klein, ed.), Vol. 4, p. 405. Springer, Vienna, 1933.

[107] Beilstein, **26,** 354 (1937).

[108] L. Ehrenberg, H. Hedström, N. Löfgren, and B. Takman, *Svensk Kem. Tid.* **58,** 269 (1946).

[109] N. Löfgren, B. Takman and H. Hedström, *Svensk Farm. Tidskr.* **53,** 321 (1949).

[110] N. Löfgren and B. Lüning, *Acta Chem. Scand.* **7,** 15 (1953).

9-β-D-ribofuranosylpurine,[111] has an inhibitory effect on tubercle bacil and mitosis in *Allium* root cells.[110] It is of interest to record that Fische predicted (1907) the natural occurrence of purine when he wrote "... (ich halte ... es nicht für unmöglich, das auch das Purin und die Methy purine im tierischen oder pflanzlichen Organismus entstehen."[112] Synthese of purine from uric acid[23] and from uracil[113] have been accomplished.

Other purines and simple purine derivatives that abound in nature ar mainly of the hydroxy and amino type. Perhaps the most widespread ar related to adenine. Adenine occurs in the free form, for example, in huma urine along with hypoxanthine and xanthine (and its methylated forms),[1 in human feces together with hypoxanthine, xanthine, and guanine,[115] an with guanine in cow's milk.[116] It is found as the free base in man plants.[117-119] It is a constituent of adenosine triphosphate (ATP), and number of coenzymes such as DPN, coenzyme A, etc.[120-125] (see Chapter 4) The thiomethylpentoside of adenine isolated from yeast,[126,127] which ha been shown by degradation and synthesis[128-131] to be 9-(5′-deoxy-5′-meth ylthio-β-D-ribofuranosyl)adenine, remained a laboratory curiosity unti its participation (in the form of a sulfonium derivative with homocysteine in transmethylation reactions[132,133] (see also Smith and Schlenk[134]) was dis-

[111] G. B. Brown and V. S. Weliky, *J. Biol. Chem.*, **204,** 1019 (1953).

[112] Ref. 9, p. 68.

[113] O. Isay, *Ber.* **39,** 250 (1906).

[114] M. Krüger and G. Salomon, *Z. physiol. Chem.* **24,** 364 (1898); **26,** 350, 389 (1898–99)

[115] M. Krüger and A. Schittenhelm. *Z. physiol. Chem.* **35,** 153 (1902).

[116] C. Voegtlin and C. P. Sherwin, *J. Biol. Chem.* **33,** 145 (1918).

[117] K. Yoshimura, *Z. physiol. Chem.* **88,** 334 (1913).

[118] A. Winterstein and F. Somló, *in* "Handbuch der Pflanzenanalyse" (Klein, ed.) Vol. 4, p. 362. Springer, Vienna, 1933.

[119] H. Bredereck, *in* "Physiologishe Chemie" (Flaschenträger and Lehnartz, eds.) Vol. 1, p. 796. Springer, Berlin, 1951.

[120] B. Lythgoe, *Ann. Repts. on Progr. Chem.* (*Chem. Soc. London*) **42,** 175 (1945).

[121] D. M. Needham, *Advances in Enzymol.* **13,** 151 (1952).

[122] *Phosphorus Metabolism,* **1** (1951).

[123] *Phosphorus Metabolism,* **2** (1952).

[124] G. W. Kenner, *Fortschr. Chem. org. Naturstoffe* **8,** 96 (1951).

[125] W. S. McNutt, *Fortschr. Chem. org. Naturstoffe* **9,** 401 (1952).

[126] J. A. Mandel and E. K. Dunham, *J. Biol. Chem.* **11,** 85 (1912).

[127] V. Suzuki, S. Odake, and T. Mori, *Biochem. Z.* **154,** 278 (1924).

[128] K. Satoh and K. Makino, *Nature* **165,** 769 (1950).

[129] F. Weygand, *Angew. Chem.* **62,** 336 (1950).

[130] J. Baddiley, O. Trauth, and F. Weygand, *Nature* **167,** 359 (1951).

[131] J. Baddiley, *J. Chem. Soc.* **1951,** 1348.

[132] G. L. Cantoni, ref. 123, p. 129.

[133] G. L. Cantoni, *J. Am. Chem. Soc.* **74,** 2942 (1952).

[134] R. L. Smith and F. Schlenk, *Arch. Biochem. and Biophys.* **38,** 159, 167 (1952).

covered by Cantoni. The antibacterial agent, cordycepin, isolated[135] from cultures of the mold *Cordyceps militaris* (Linn.) Link, is[136] an adenine-9-nucleoside of cordycepose, a branched 3-deoxypentose related structurally to apiose. Adenine is present in pseudovitamin B_{12}, thus replacing 5,6-dimethylimidazole in the nucleotide portion of B_{12}.[137] An antibiotic, Puromycin, occurring in the mold *Streptomyces alboniger*, has been found to be active against certain bacteria and Trypanosomes;[138] upon acid hydrolysis it yields the dimethyladenine, 6-dimethylaminopurine, and D-3-aminoribose and *O*-methyl-L-tyrosine.[139] In its proposed structure, Puromycin is shown[139] as a 9-[*O*-methyl-L-tyrosyl-*N*-3′-aminoribosyl]-6-dimethylaminopurine. 6-Dimethylaminopurine has previously been synthesized by the reaction of 6-methylmercaptopurine with dimethylamine.[140]

In addition to its presence in the free form in bird droppings and other sources already mentioned, guanine has an interesting distribution in nature. It accounts for the iridescence of fish scales[141-143] and as such in many teleosts is an inexpensive source of "pearl essence."[142, 144] It was used in 1656 in France to impart a pearly appearance to beads.[145] Guanine is found in the eyes of the dogfish[146] (cf. Hopkins[147]), in the excreta of spiders,[148] in sugar and refuse molasses,[149] and (with xanthine) in the shoots of a food-herb (*Aralia cordata*) commonly used in Japan.[150] The white, shiny appearance of the skin of many amphibia and reptiles is due to the presence of guanine (see Abderhalden, "Biochemisches Handlexikon," in the General References section).

Guanine accumulates[151] in large crystalline masses in the bones and other tissues of the pig in a metabolic disorder[152] that bears a strong resemblance to human gout. The absence of guanase in swine[153] and of uricase in the human[154] and the very low sol bility of guanine and uric acid (mono sodium salt) serve to explain, in part, the acc mulation of these purines often seen in gout (see Gutman[155] Bauer and Klemperer for an extensive description of human gout and the participation of uric acid in th disease). Uric acid gout also affects the bird.[157] Originally mistaken for uric acid, the extremely insoluble 2,8-dihydroxyadenine (XXIII)[29, 159] deposits in crystalli condition in the kidney tubules of the rat[160, 161] following massive administration adenine.

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[135] K. G. Cunningham, S. A. Hutchinson, W. Manson, and F. S. Spring, *J. Chem. Soc.* **1951,** 2299.

[136] H. R. Bentley, K. G. Cunningham, and F. S. Spring, *J. Chem. Soc.* **1951,** 2301.

[137] H. W. Dion, D. G. Calkins, and J. J. Pfiffner, *J. Am. Chem. Soc.* **74,** 1108 (1952).

[138] J. N. Porter, R. I. Hewitt, C. W. Hesseltine, G. Krupka, J. A. Lowery, W. S. Wallace, N. Bohonos, and J. H. Williams, *Antibiotics & Chemotherapy* **2,** 409 (1952).

[139] C. W. Waller, P. W. Fryth, B. L. Hutchings, and J. H. Williams, *J. Am. Chem. Soc.* **75,** 2025 (1953).

[140] G. B. Elion, E. Burgi, and G. H. Hitchings, *J. Am. Chem. Soc.* **74,** 411 (1952).

[141] Barreswill, *Ann.* **122,** 128 (1862).

[142] A. Bethe, *Z. physiol. Chem.* **20,** 472 (1895).

[143] G. H. Hitchings and E. A. Falco, *Proc. Natl. Acad. Sci. U. S.* **30,** 294 (1944).

[144] D. K. Tressler and J. MacW. Lemon, "Marine Products of Commerce," 2nd ed., p. 117. Reinhold, New York, 1951.

[145] H. F. Taylor, *in* "Marine Products of Commerce" (D. K. Tressler, ed.), p. 161. Chemical Catalog Co. New York, 1923.

[146] A. Pirie and D. M. Simpson, *Biochem. J.* **40,** 14 (1946).

[147] F. G. Hopkins, *Proc. Roy. Soc.* (*London*) **B130,** 359 (1941–42).

[148] E. Gorup-Besanez and F. Will, *Ann.* **67,** 117 (1849).

[149] E. C. Shorey, *J. Am. Chem. Soc.* **21,** 609 (1899).

[150] K. Miyake, *J. Biol. Chem.* **21,** 507 (1915).

[151] R. Virchow, *Arch. pathol. Anat. u. Physiol.* (*Virchow's*) **35,** 358 (1866); **36,** 147 (1866).

[152] W. Mendelson, *Am. J. Med. Sci.* **95,** 109 (1888).

Uric acid (XXIV)[162] has received a much more extensive treatment the literature than any other purine[9-11,13,20,118,119,163] (cf. also General Re erences). It is the chief end-product of purine (and protein) metabolism in great many, but not all, animal species. Perhaps less well known is its pre ence in plants[164,165] and in the wings of certain butterflies.[147, 166, 167] In add tion to its distribution in a variety of body fluids, uric acid is also found large amounts as a D-riboside in beef and human red corpuscles[168-170] and traces in those of other animal species. On the basis of ultraviolet spectra properties, the ribose radical of a uric acid riboside from beef blood an liver was suggested to be situated at position 9 of the purine.[171] Anothe preparation of uric acid riboside from beef erythrocytes possesses differen spectral properties.[172] For a synthesis of uric acid, see below.

In 1897, Fischer transformed uric acid into isoguanine (XXV), or 2 hydroxyadenine) and considered it not unlikely[29] that the latter might b an animal product. The prediction appeared to have been borne out by the reported isolation of isoguanine from pig blood,[173] although this finding could not be confirmed[174] (see also Schütz[175]). However, isoguanine occurs in the wings of butterflies[176] and was once[177] thought to be a pterin ("guanopterin"). Isoguanine is also found as the aglycone of the riboside, crotonoside, in the croton bean (*Croton tiglium* L.).[178,179] The structure of crotonoside as a 9-riboside of isoguanine was inferred from ultraviolet absorption spectra,[180] and established as 9-β-D-ribofuranosylisoguanine by synthesis.[181] A total synthesis of isoguanine has been accomplished.[182]

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[153] W. Jones and C. R. Austrian, *Z. physiol. Chem.* **48,** 110 (1906).

[154] W. D. Geren, A. Bendich, O. Bodansky, and G. B. Brown, *J. Biol. Chem.* **183,** 2 (1950).

[155] A. B. Gutman, *Am. J. Med.* **9,** 799 (1950).

[156] W. Bauer and F. Klemperer, *in* "Diseases of Metabolism" (Duncan, ed.), 3rd ed. p. 683. Saunders, Philadelphia, 1952.

[157] B. F. Kaupp, "Poultry Diseases," 4th ed., p. 228. Alexander Eger, Chicago, 1927

[158] O. Minkowski, *Arch. exptl. Pathol. Pharmakol.* **41,** 375 (1898).

[159] A. Nicolaier, *Z. klin. Med.* **45,** 359 (1902).

[160] A. Bendich, G. B. Brown, F. S. Philips, and J. B. Thiersch, *J. Biol. Chem.* **183,** 26 (1950).

[161] F. S. Philips, J. B. Thiersch, and A. Bendich, *J. Pharmacol. Exptl. Therap.* **104,** 2 (1952).

[162] Beilstein, **26,** 513 (1937).

[163] H. Biltz, "Die neuere Harnsäurechemie." J. A. Barth, Leipzig, 1936. (Reprinted fro *J. prakt. Chem.* [2] **145,** 65 (1936).)

[164] M. Sumi, *Biochem. Z.* **195,** 161 (1928).

[165] R. Fosse, P. DeGraeve, and P. Thomas, *Compt. rend.* **194,** 1408 (1932).

[166] V. B. Wigglesworth, *Proc. Roy. Soc.* (*London*) **B97,** 149 (1925).

[167] A. Tarttar, *Z. physiol. Chem.* **266,** 130 (1940).

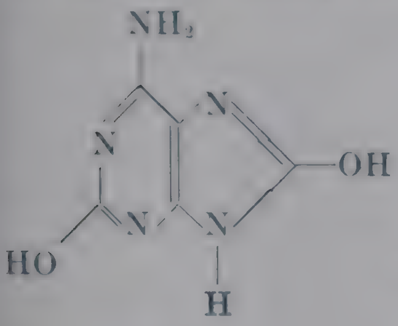
[168] A. R. Davis, E. B. Newton, and S. R. Benedict, *J. Biol. Chem.* **54,** 595 (1922).

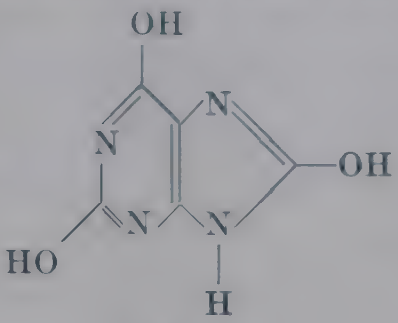
[169] E. B. Newton and A. R. Davis, *J. Biol. Chem.* **54,** 601 (1922).

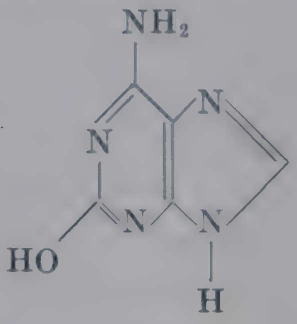
[170] E. B. Newton and A. R. Davis, *J. Biol. Chem.* **54,** 603 (1922).

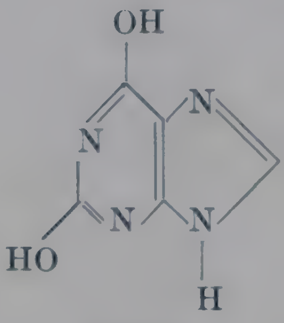
[171] R. Falconer and J. M. Gulland, *J. Chem. Soc.* **1939,** 1369.

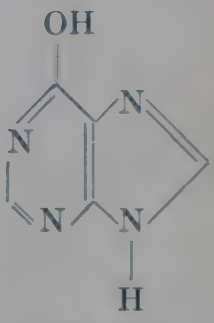
[172] C. E. Carter and J. L. Potter, *Federation Proc.* **11,** 195 (1952).


XXIII
2,8-Dihydroxyadenine


XXIV
Uric acid


XXV
Isoguanine


XXVI
Xanthine


XXVII
Hypoxanthine

Reference to xanthine (XXVI)[183] and hypoxanthine or "sarkin" (XXVII)[184] has been made a number of times above. These substances are frequently present in animal sources as a result of the action of the enzymes guanase and adenase on the parent purines or of nucleosidases on the corresponding nucleoside. Xanthine was discovered by Marcet (1817) in bladder stones.[185] Hypoxanthine was found in beef spleen (and so named by Scherer[186] in 1850), in beef and horse meat, and in creatine mother-liquors by Strecker.[187] The distribution of hypoxanthine and xanthine (and its methylated derivatives such as caffeine, theophylline, and theobromine) in tea,[188] coffee, and cocoa and as urinary constituents is ably discussed elsewhere.[189,190] A purine nucleoside, spongosine, isolated from the sponge *Cryptotethia crypta* (Florida), has been shown to be a pentosylmethylaminoöxypurine, the aglycone of which has been deaminated with nitrous acid to a methyldioxypurine.[191]

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[173] M. V. Buell and M. E. Perkins, *J. Biol. Chem.* **72,** 745 (1927).

[174] S. Bergström, P. Edman, and O. Hall, *Acta Chem. Scand.* **3,** 1128 (1949).

[175] F. A. Schütz, *Biochem. Z.* **273,** 52 (1934).

[176] R. Purrmann, *Ann.* **544,** 182 (1940).

[177] C. Schöpf and E. Becker, *Ann.* **524,** 49 (1936).

[178] E. Cherbuliez and K. Bernhard, *Helv. Chim. Acta* **15,** 464, 978 (1932).

[179] J. R. Spies, *J. Am. Chem. Soc.* **61,** 350 (1939).

[180] R. Falconer, J. M. Gulland, and L. F. Story, *J. Chem. Soc.* **1939,** 1784

[181] J. Davoll, *J. Am. Chem. Soc.* **73,** 3174 (1951).

[182] A. Bendich, J. F. Tinker, and G. B. Brown, *J. Am. Chem. Soc.* **70,** 3109 (1948).

[183] Beilstein, **26,** 447 (1937).

[184] Beilstein, **26,** 416 (1937).

The free purines and their simple derivatives have been found much more frequently in nature than the pyrimidines. Of the nucleic acid pyrimidines, only uracil has been found in the free form,[63] and recently hydrouracil (2,6-dihydroxy-4,5-dihydropyrimidine) has been obtained from beef spleen.[192] A pentofuranoside of thymine, spongothymidine, thought to be a xylofuranoside, was isolated[191,193] from Florida *Cryptotethia* sponges. The identity of the sugar portion as xylose was supported by paper chromatographic analysis,[194] and the point of its attachment to N-3 of the thymine follows from the resistance of the glycoside towards acid hydrolysis[191] and from the character of the ultraviolet absorption spectra.[191,195]

The coenzyme of the system (galactowaldenase) which catalyzes the conversion of galactose-1-phosphate into glucose-1-phosphate[196] has been isolated from bakers' yeast;[197,198] the coenzyme contains uridine, two phosphate groups, and glucose and is named uridine diphosphate glucose (UDPG). Upon gentle acid hydrolysis of UDPG, glucose and a uridine diphosphate (shown by synthesis to be uridine-5′-pyrophosphate[199]) were obtained and the formula XXVIII was assigned;[196-198] the formula

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[185] Marcet, "An Essay on the Chemical History and Medical Treatment of Calcul Disorders," London, 1817: cited in refs. 18, 119, and 183.

[186] J. Scherer, *Ann.* **73,** 328 (1850).

[187] A. Strecker, *Ann.* **102,** 204 (1857); **108,** 129 (1858).

[188] A. Baginsky, *Z. physiol. Chem.* **8,** 395 (1883–84).

[189] W. C. Rose, *Physiol. Revs.* **3,** 544 (1923).

[190] A. A. Christman, *Physiol. Revs.* **32,** 303 (1952).

[191] W. Bergmann and R. J. Feeney, *J. Org. Chem.* **16,** 981 (1951).

[192] C. Funk, A. J. Merritt, and A. Ehrlich, *Arch. Biochem. and Biophys.* **35,** 468 (1952).

[193] W. Bergmann and R. J. Feeney, *J. Am. Chem. Soc.* **72,** 2809 (1950).

[194] K. Makino and K. Satoh, *Abstr. 12th Intern. Congr. Pure Appl. Chem.* **1951,** 317.

[195] J. J. Fox and D. Shugar, *Biochim. et Biophys. Acta* **9,** 369 (1952).

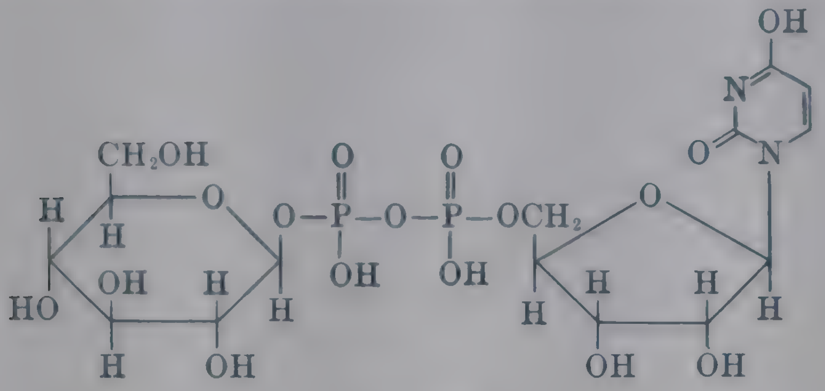
[196] L. F. Leloir, ref. 122, p. 67.

[197] C. E. Cardini, A. C. Paladini, R. Caputto, and L. F. Leloir, *Nature* **165,** 191 (1950).

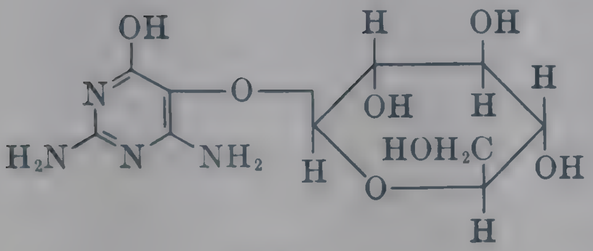
[198] R. Caputto, L. F. Leloir, C. E. Cardini, and A. C. Paladini, *J. Biol. Chem.* **184,** 333 (1950).

[199] N. Anand, V. M. Clark, R. H. Hall, and A. R. Todd, *J. Chem. Soc.* **1952,** 3665.

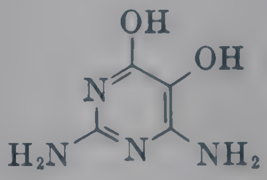
(XXVIII) given here is arbitrary in that the configuration of the glucose portion is not yet known. Three nucleotide derivatives, all of which include uridine-5′-pyrophosphate attached to an aminosugar by an acetal-like bond, accumulate in penicillin-treated cultures of *S.* Aureus;[200,201] one of these substances contains an L-alanine residue and another contains a peptide composed of L-lysine, D-glutamic acid, and three alanine residues.



XXVIII
Uridine diphospate glucose



XXIX
Vicine



XXX
Divicine

Vicine ($C_{10}H_{16}O_7N_4$) was isolated from legumes of the species *vicia* in 1870 by Ritthausen[202-204] and appears to be the first simple pyrimidine derivative found in nature. It also occurs[118] in beet juice[205] and peas.[206] Upon mild acid hydrolysis, it yields the aglucone divicine ($C_4H_6O_2N_4$)[203,204] and D-glucose.[207,208] Johnson[14,18,209,210] suggested a "pyrimidine-nucleoside" structure for vicine. Levene found[13,211,212] that half of the nitrogen of vicine

[200] J. T. Park, ref. 122, p. 93.
[201] J. T. Park, *J. Biol. Chem.* **194,** 877, 885, 897 (1952).
[202] H. Ritthausen and V. Kreusler, *J. prakt. Chem.* [2] **2,** 333 (1870).
[203] H. Ritthausen, *J. prakt. Chem.* [2] **7,** 374 (1873); **59,** 480, 482 (1899).
[204] H. Ritthausen, *Ber.* **9,** 301 (1876); **29,** 2108 (1896).
[205] E. O. von Lipmann, *Ber.* **29,** 2653 (1896).
[206] E. Schultz, *Z. physiol. Chem.* **15,** 140 (1890); **17,** 215 (1893).
[207] E. Fischer, *Ber.* **47,** 2611 (1914).
[208] H. Hérissey and J. Cheymol, *Bull. soc. chim. biol.* **13,** 29 (1931).
[209] T. B. Johnson, *J. Am. Chem. Soc.* **36,** 337 (1914).
[210] T. B. Johnson and C. O. Johns, *J. Am. Chem. Soc.* **36,** 545 (1914`.
[211] P. A. Levene, *J. Biol. Chem.* **18,** 305 (1914).
[212] P. A. Levene and J. K. Senior, *J. Biol. Chem.* **25,** 607 (1916).

as well as of divicine was present as free amino groups, one of which was part of a guanidino system, and he concluded that vicine was a 3-*N*-glucoside of 2,5-diamino-4,6-dihydroxypyrimidine. Recent evidence[213] has shown this widely accepted formulation to be incorrect. Vicine was assigned the structure XXIX (i.e., 2,4-diamino-6-hydroxy-5-(β-D-glucopyranoside)) and divicine that of 2,4-diamino-5,6-dihydroxypyrimidine (XXX) on the basis of a study of ultraviolet absorption spectra and a variety of chemical properties.[213,214] Divicine belongs to a group of pyrimidines and other compounds (such as ascorbic acid) that contain an enediol, aminoenol, or amino-eneamine system conjugated to a carbonyl (or potential carbonyl) group and therefore possess strong reducing properties;[214] vicine is non-reducing. A similar substance, convicine ($C_{10}H_{15}O_8N_3 \cdot H_2O$), accompanies vicine in *vicia*.[215,216] Convicine is believed to be a hexoside of 4-amino-2,5,6-trihydroxypyrimidine,[217] and its chemical properties[215-217] indicate that this glycoside is very closely related to vicine in structure.

A compound of increasing interest and importance, orotic acid[218] (XXXI, or uracil-4-carboxylic acid) was discovered in the whey of cow's milk by Biscaro and Belloni[219] in 1905; the name is derived from the Greek: *oros* = whey. It is found in the milk of sheep and goats, and in smaller amounts in human, pig, and horse milk,[220-222] and has been isolated from "distillers dried solubles."[223] Orotic acid accumulates in large quantities during the growth of mutants of the fungus *Neurospora*, which require uridine, cytidine, or uracil,[224] and is required for the growth of *L. bulgaricus* 09.[220,221] For a discussion of the participation of orotic acid in nucleic acid pyrimidine biosynthesis see Chapters 23 and 25. A riboside of orotic acid (orotidine) has also been isolated[225] from *Neurospora*; unlike uridine and cytidine, orotidine hydrolyzes in dilute mineral acid as readily as do the ribosides of the purines.

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[213] A. Bendich, *Trans. N. Y. Acad. Sci.*, [2] **15,** 58 (1952).

[214] A. Bendich and G. C. Clements, *Biochim. et Biophys. Acta* **12,** 462 (1953).

[215] H. Ritthausen, *J. prakt. Chem.* [2] **24,** 202 (1881).

[216] H. Ritthausen and Dr. Preuss, *J. prakt. Chem.* [2] **59,** 489 (1899).

[217] H. J. Fischer and T. B. Johnson, *J. Am. Chem. Soc.* **54,** 2038 (1932).

[218] Beilstein, **26,** 253 (1936).

[219] G. Biscaro and E. Belloni, *Ann. Soc. Chim. Milano* **11,** 18, 71 (1905); a fairly detailed account of this work given in *Chem. Centr.* **II, 76,** 63 (1905).

[220] O. P. Wieland, J. Avener, E. M. Boggiano, N. Bohonos, B. L. Hutchings, and J. H. Williams, *J. Biol. Chem.* **186,** 737 (1950).

[221] J. W. Huff, D. K. Bosshardt, L. D. Wright, D. S. Spicer, K. A. Valentik, and H. R. Skeggs, *Proc. Soc. Exptl. Biol. Med.* **75,** 297 (1950); see also *J. Am. Chem. Soc.* **72,** 2312 (1950).

[222] L. E. Hallanger, J. W. Laasko, and M. O. Schultze, *J. Biol. Chem.* **202,** 83 (1953).

[223] L. Manna and S. M. Hauge, *J. Biol. Chem.* **202,** 91 (1953).

[224] H. K. Mitchell, M. B. Houlahan, and J. F. Nyc, *J. Biol. Chem.* **172,** 525 (1948).

[225] A. M. Michelson, W. Drell, and H. K. Mitchell, *Proc. Natl. Acad. Sci. U. S.* **37,** 396 (1951).

Biscaro and Belloni degraded orotic acid to urea with permanganate and proposed a 7-membered cyclic ureido structure for their compound.[219] Wheeler *et al.*[226] pointed out that the empirical formula of orotic acid corresponded to that of a carboxyuracil, and they synthesized the 5-carboxylic acid only to find it different from orotic acid. Wheeler also synthesized[227] uracil-4-carboxylic acid, but its melting point (347°, dec.) was not the same as that (260°, dec.) reported[219] for orotic acid. In a reconsideration of this problem twenty-two years later, Johnson and Caldwell described a synthesis of 2,5-dihydroxypyrimidine-4-carboxylic acid; its melting point, 259°, led them to believe that it was identical with orotic acid.[228] It remained for Bachstez[229] to prove the identity of orotic acid when he compared a specimen (prepared by Biscaro) which now melted at 345–6° (dec.) with uracil-4-carboxylic acid previously synthesized by Wheeler[227] and by Behrend and Struve.[230] Although Wheeler did in fact synthesize XXXI,[227] he did not realize that XXXI had resulted from the alkaline hydrolysis and rearrangement of 5-(carbethoxymethylidene)hydantoin (XXXII, prepared from oxalacetic ethyl ester and urea by Müller's[231] method) rather than (as he thought) from the saponification of the ethyl ester of XXXI. For the clarification and a discussion of these points the reader is referred to the interesting work of Mitchell and Nyc.[232,233] In the other early synthesis,[230] 4-methyluracil (XVIII) was converted to orotic acid upon alkaline ferricyanide oxidation. Thymine is not oxidized (see Johnson and Schroeder[234]) to the isomeric 5-carboxylic acid.

An improved synthesis[234] of orotic acid (which has been adapted[235] for the introduction of C^{14} in position 6) is shown below together with the reactions already discussed.

c. Purine and Pyrimidine Derivatives and Analogues of Biological Importance

The early investigators of purines and pyrimidines could hardly have suspected that what appeared as academic exercises in pure chemistry would furnish a broad foundation for future biological thought and en-

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[226] H. L. Wheeler, T. B. Johnson, and C. O. Johns, *Am. Chem. J.* **37,** 392 (1907).

[227] H. L. Wheeler, *Am. Chem. J.* **38,** 358 (1907).

[228] T. B. Johnson and W. T. Caldwell, *J. Am. Chem. Soc.* **51,** 873 (1929).

[229] M. Bachstez, *Ber.* **63,** 1000 (1930).

[230] R. Behrend and K. Struve, *Ann.* **378,** 153 (1911).

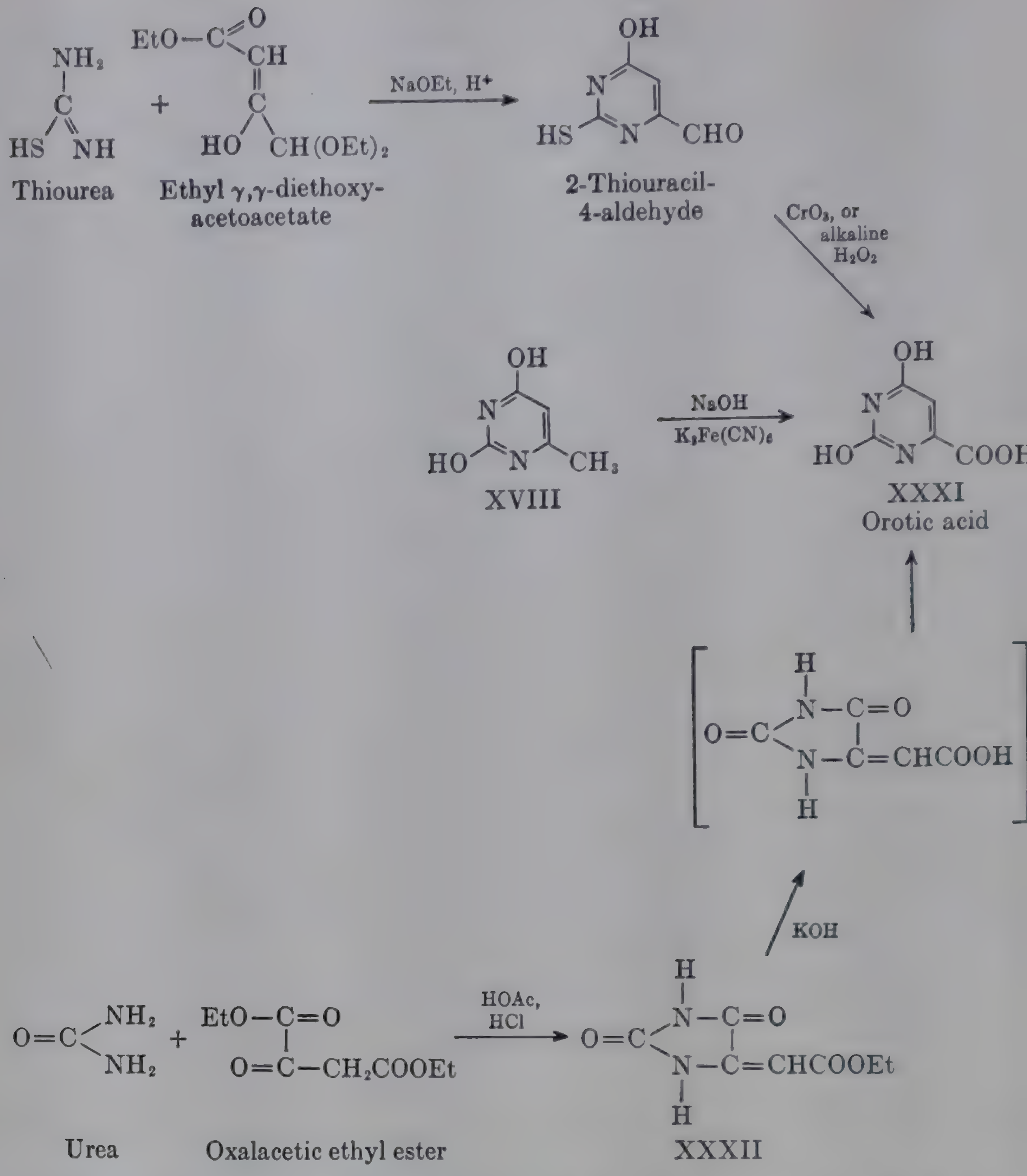
[231] R. Müller, *J. prakt. Chem.* [2] **55,** 505; **56,** 475 (1897).

[232] H. K. Mitchell and J. F. Nyc, *J. Am. Chem. Soc.* **69,** 674 (1947).

[233] J. F. Nyc and H. K. Mitchell, *J. Am. Chem. Soc.* **69,** 1382 (1947).

[234] T. B. Johnson and E. F. Schroeder, *J. Am. Chem. Soc.* **53,** 1989 (1931).

[235] C. Heidelberger and R. B. Hurlbert, *J. Am. Chem. Soc.* **72,** 4704 (1950).



deavor. Examples shall be cited here of the contributions made by thes workers which have strongly influenced modern medicine. Mention ha already been made, for instance, of uric acid and gout. Brugnatelli[2] obtained alloxan (2,6-dihydroxypryimidine-4,5-dione) in 1818 from th nitric acid oxidation of uric acid. The Wöhler and Liebig preparation c alloxan[4] is well known. In 1943, the important discovery was made[23 that the administration of alloxan to rabbits leads to a necrosis of th insulin-producing units of the pancreas (the beta-cells of the islets c Langerhans), thereby providing a tool for the production and study c experimental diabetes. Alloxan is diabetogenic in many animals includin

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[236] J. S. Dunn, H. L. Sheehen, and N. G. B. McLetchie, *Lancet* i, 484 (1943).

man (see Bailey[237] and Lazarow[238]). In 1864, Baeyer[5] transformed alloxan into barbituric acid[239] (2,4,6-trihydroxypyrimidine), the total synthesis of which (from urea and malonic acid) was carried out by Grimaux fourteen years later.[240] The barbiturates are the best known of the hypnotics and sedatives; barbital (5,5-diethylbarbituric acid) and phenobarbital (5-ethyl-5-phenylbarbituric acid) have been in popular use since the first of the two was introduced[241] into clinical practice in 1903 (see also Goodman and Gilman[242]).

A single change in the structure of uracil, i.e., the substitution of its 2-hydroxy by mercapto, gives rise to a compound, 2-thiouracil[243-245] which possesses several important biological properties. For example, 2-thiouracil and certain of its 4-alkyl derivatives are effective in the treatment of hyperthyroidism and thyrotoxicosis in man.[246-248] Studies of antithyroid activity and structure of substituted thiouracils have been made,[249,250] and it has been suggested that these compounds may prevent iodination of thyroxine precursors.[251] The mode of action of these drugs is still a mystery. Better understood, however, is the 2-thiouracil inhibition of *E. coli*, since this bacteriostatic action is prevented by uracil.[252] Uracil also causes a reversal of the 2-thiouracil inhibition of tobacco mosaic virus biosynthesis.[253] Thiouracil may therefore be classified as an antimetabolite and its antagonism by uracil may be a reflection either of the role of uracil as a component of certain enzyme systems (see above) or of the nucleic acids. (For a discussion of antimetabolites and chemotherapy, see the literature[254-257].)

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[237] C. C. Bailey, *Vitamins and Hormones* **7,** 365 (1949).

[238] A. Lazarow, *Physiol. Revs.* **29,** 48 (1949).

[239] Beilstein, **24,** 467 (1936).

[240] M. E. Grimaux, *Bull. soc. chim.* (Nouv. Sér.) **31,** 146 (1879); *Compt. rend.* **87,** 752 (1878).

[241] E. Fischer and v. Mering, *Chem. Centr.* **74, I,** 1155 (1903).

[242] L. Goodman and A. Gilman, "The Pharmacological Basis of Therapeutics," p. 126. Macmillan, New York, 1941.

[243] Beilstein, **24,** 323 (1936).

[244] H. L. Wheeler and H. S. Bristol, *Am. Chem. J.* **33,** 448 (1905).

[245] H. L. Wheeler and L. M. Liddle, *Am. Chem. J.* **40,** 547 (1908).

[246] E. B. Astwood, *J. Am. Med. Assoc.* **122,** 78 (1943); *Harvey Lectures* **40,** 195 (1945).

[247] H. P. Himsworth, *Lancet* ii, 465 (1943); see also p. 483.

[248] W. W. van Winkle, Jr., S. M. Hardy, G. R. Hazel, D. C. Hines, H. S. Newcomer E. A. Sharp, and W. N. Sisk, *J. Am. Med. Assoc.* **130,** 343 (1946).

[249] G. W. Anderson, I. F. Halverstadt, W. H. Miller, and R. O. Roblin, Jr., *J. Am. Chem. Soc.* **67,** 2197 (1945).

[250] R. H. Williams and G. A. Kay, *Am. J. Med. Sci.* **213,** 198 (1947).

[251] W. H. Miller, R. O. Roblin, Jr., and E. B. Astwood, *J. Am. Chem. Soc.* **67,** 2201 (1945).

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[253] B. Commoner and F. Mercer, *Nature* **168,** 113 (1951); *Arch. Biochem. and Biophys.* **35, 278** (1952).

Other pyrimidines active as microbial inhibitors have also been known for mar years, and include 5-methylthiouracil,[252] dithiothymine, and 5-hydroxy-, 5-amino 5-halo-, and 5-nitrouracils.[258-260] Certain 2,6(4)-diaminopyrimidines containing grou such as benzyl, phenoxy, or phenyl at position 5 are powerful antagonists of folic folinic acids in a variety of biological systems[261] and inhibit the development of fro eggs.[262] (For a discussion of these acids and purine and pyrimidine biosynthesis, se Shive.[262a]) The structural resemblance between the antimalarial Paludrine (N^1- chlorophenyl-N^5-isopropylbiguanide) and certain of these 2,6(4)-diamino-5-aryloxy pyrimidines led to the suggestion that Paludrine might also possess anti-folic activit and that these pyrimidines might be effective antimalarials.[263] These consideratior were confirmed upon experimentation, and many active derivatives were prepared.[264,2 One of the most effective is 2,6(4)-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidin (Daraprim),[266,267] which is about 1000 times more active than quinine and about 2 times more active than Paludrine. It is of interest that the open-chain Paludrine wa originally designed from a study of the active structural moiety of active 2-(pheny guanidino)pyrimidines.[268] Other examples of pyrimidines used in chemotherapy ar the pyrimidine sulfonamides.[269]

Just as in the cases of the pyrimidines cited above, alteration of th structures of naturally occurring purines has yielded compounds of valu in biology and medicine. The changes that have furnished important ana logues to be discussed here have involved substituents at C-2 and C-6 a well as the replacement of carbons 2 and 8 by nitrogen. The 2-amino substituted adenine, 2,6-diaminopurine or DAP[182,270] has been found t

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[254] R. O. Roblin, Jr., *Chem. Revs.* **38,** 255 (1946).

[255] L. D. Wright, *Vitamins and Hormones* **9,** 131 (1951).

[256] D. W. Woolley, "A Study of Antimetabolites." Wiley, New York, 1952.

[257] A. Albert, "Selective Toxicity with Special Reference to Chemotherapy." Methuer London, 1951.

[258] G. H. Hitchings, G. B. Elion, and E. A. Falco, *J. Biol. Chem.* **185,** 643 (1950).

[259] G. H. Hitchings, G. B. Elion, E. A. Falco, P. B. Russell, and H. VanderWerff, *Anr N. Y. Acad. Sci.* **52,** 1318 (1950).

[260] G. H. Hitchings, G. B. Elion, E. A. Falco. P. B. Russell, M. B. Sherwood, and H VanderWerff, *J. Biol. Chem.* **183,** 1 (1950).

[261] G. H. Hitchings, E. A. Falco, H. VanderWerff, P. B. Russell, and G. B. Elion, *J Biol. Chem.* **199,** 43 (1952).

[262] S. Bieber, R. F. Nigrelli, and G. H. Hitchings, *Proc. Soc. Exptl. Biol. Med.* **79,** 43 (1952).

[262a] W. Shive, *Vitamins and Hormones* **9,** 75 (1951).

[263] E. A. Falco, G. H. Hitchings, P. B. Russell, and H. VanderWerff, *Nature* **164,** 10 (1949).

[264] L. G. Goodwin, *Nature* **164,** 1133 (1949).

[265] E. A. Falco, P. B. Russell, and G. H. Hitchings, *J. Am. Chem. Soc.* **73,** 3753 (1951

[266] P. B. Russell and G. H. Hitchings, *J. Am. Chem. Soc.* **73,** 3763 (1951).

[267] L. H. Schmidt and C. S. Genther, *J. Pharmacol. Exptl. Therap.* **107,** 61 (1953).

[268] H. R. Ing, *in* "Organic Chemistry, An Advanced Treatise" (Gilman, ed.), Vol. p. 392. Wiley, New York, 1953.

[269] E. H. Northey, "The Sulfonamides and Allied Compounds," p. 31. Reinhold, Ne York, 1948.

[270] W. Traube, *Ber.* **37,** 4544 (1904).

serve as a precursor of the guanine of nucleic acids of the rat[271] and the purines of those of other species (see Chapters 23 and 25). Earlier studies, however, revealed DAP to be a potent inhibitor of *L. casei*; this inhibition could be reversed by adenine.[269, 272, 273] Many other effects of DAP have been recorded (summarized in Wright[255]), and these include a prolongation of the life of leukemic mice[274, 275] and damage (which is prevented by adenine) to sarcoma cells in tissue culture[276] and to the hematopoietic apparatus and intestinal epithelium of small mammals.[277] DAP affects a number of biological systems rich in nucleic acids: it inhibits the multiplication of vaccinia virus in chick embryonic tissue,[278] of Russian Spring Summer Encephalitis virus *in vitro*[279] and *in vivo*,[280] and of kappa particles in killer *Paramecium aurelia*.[281, 282] These effects are reversed by adenine. Other inhibitory adenines are the 2-chloro,[283] 2-thio, and 2-ethylthio derivatives.[284]

The ultimate replacement of the amino group of adenine by mercapto has been accomplished by the treatment of hypoxanthine with phosphorus pentasulfide.[140] The resulting 6-mercaptopurine, which inhibits the growth of *L. casei*,[284, 285a] possesses the unique and important property of rendering a malignant tumor (mouse sarcoma 180) nonviable.[285b] It has a marked inhibitory effect on many mouse and rat tumors;[285c] it shows a toxicity to mammals that is suggestive of an antagonism of polynucleotide biosynthesis;[285d] and results of preliminary application to human neoplastic ease[285e] have stimulated further clinical trials with this drug.

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[271] A. Bendich, S. S. Furst, and G. B. Brown, *J. Biol. Chem.* **185,** 423 (1950).

[272] G. H. Hitchings, G. B. Elion, H. VanderWerff, and E. A. Falco, *J. Biol. Chem.* **174,** 765 (1948).

[273] G. B. Elion and G. H. Hitchings, *J. Biol. Chem.* **187,** 511 (1950).

[274] J. H. Burchenal, A. Bendich, G. B. Brown, G. B. Elion, G. H. Hitchings, C. P. Rhoads, and C. C. Stock, *Cancer* **2,** 119 (1949).

[275] H. E. Skipper, L. L. Bennett, Jr., P. C. Edwards, C. E. Bryan, O. S. Hutchison, J. B. Chapman, and M. Bell, *Cancer Research* **10,** 166 (1950).

[276] J. J. Biesele, R. E. Berger, A. Y. Wilson, G. H. Hitchings, and G. B. Elion, *Cancer* **4,** 186 (1951).

[277] F. S. Philips and J. B. Thiersch, *Proc. Soc. Exptl. Biol. Med.* **72,** 401 (1949).

[278] R. L. Thompson, M. L. Price, S. A. Minton, Jr., G. B. Elion, and G. H. Hitchings, *J. Immunol.* **65,** 529 (1950).

[279] C. Friend, *Proc. Soc. Exptl. Biol. Med.* **78,** 150 (1951).

[280] A. E. Moore and C. Friend, *Proc. Soc. Exptl. Biol. Med.* **78,** 153 (1951).

[281] W. E. Jacobson, M. Williamson, and C. C. Stock, *J. Exptl. Zool.* **121,** 505 (1952).

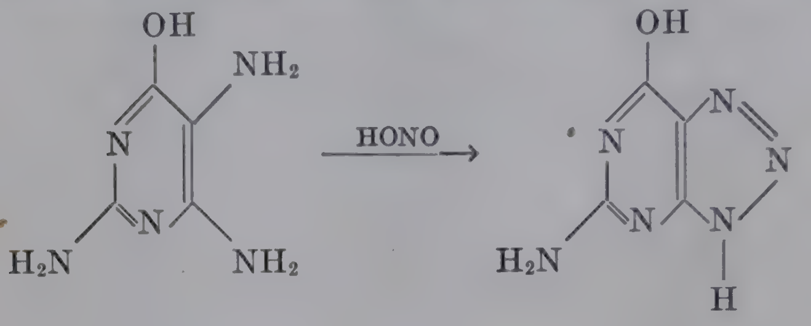
[282] M. Williamson, W. Jacobson, and C. C. Stock, *J. Biol. Chem.* **197,** 763 (1952).

[283] J. J. Biesele, R. E. Berger, and M. Clarke, *Cancer Research* **12,** 465 (1952).

[284] G. B. Elion, G. H. Hitchings, and H. VanderWerff, *J. Biol. Chem.* **192,** 505 (1951).

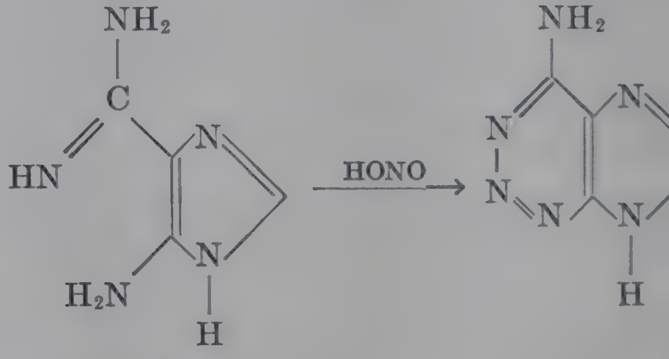
[285] *Proc. Amer. Assoc. Cancer Research* **1,** 1953;
(a) G. B. Elion and G. H. Hitchings, p. 13.
(b) D. A. Clarke, F. S. Philips, S. S. Sternberg, C. C. Stock, and G. B. Elion, p. 9.
(c) K. Sugiura, p. 55.
(d) F. S. Philips, S. S. Sternberg, D. A. Clarke, and G. H. Hitchings, p. 42.
(e) J. H. Burchenal, D. A. Karnofsky, L. Murphy, R. R. Ellison, and C. P. Rhoads, p. 7.

In 1901, Gabriel and Colman[286] converted 4,5-diamino-6-methylpyri dine into 6-methyl-8-azapurine (or 7-methyl-1-*v*-triazolo[*d*]pyrimidi upon reaction with nitrous acid. The isosteric relationship of this compou to 6-methylpurine[286] is obvious. With the expectation of preparing pur antagonists, Roblin *et al.*[287] applied the above reaction to the appropri 4,5-diaminopyrimidines and obtained the 8-aza analogues of adenine, guanine (XXXIII, or "guanazolo"), and of hypoxanthine. Those of adeni and guanine exhibited an antibacterial activity (against *E. coli* and



2,4,5-Triamino-6-hydroxypyrimidine

XXXIII
8-Azaguanine



4-Amino-5-imidazole-carboxamide

XXXIV
2-Azaadeni

aureus), and this action was reversed by the respective parent purin Other 8-azapurines have been synthesized,[288,289] but 8-azaguanine h provoked the most interest. It inhibits the growth of the guanine-requiri protozoan *T. geleii*,[290] the mammary adenocarcinoma E_0 771 in C_{57} bla mice,[291,292] and the development of several plant virus infections.[293]

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[286] S. Gabriel and J. Colman, *Ber.* **34,** 1234 (1901).

[287] R. O. Roblin, Jr., J. O. Lampen, J. P. English, Q. P. Cole, and J. R. Vaughan, *Am. Chem. Soc.* **67,** 290 (1945).

[288] L. F. Cavalieri, A. Bendich, J. F. Tinker, and G. B. Brown, *J. Am. Chem. Soc.* 3875 (1948).

[289] P. Bitterli and H. Erlenmeyer, *Helv. Chim. Acta*, **34,** 835 (1951).

[290] G. W. Kidder and V. C. Dewey, *J. Biol. Chem.* **179,** 181 (1949).

[291] G. W. Kidder, V. C. Dewey, R. E. Parks, Jr., and G. L. Woodside, *Science* **109,** (1949).

clue to the mechanism of the inhibitory activity of this agent (and possibly others) is furnished by the reports of its apparent incorporation into the nucleic acids of *T. geleii*[294] and the normal and tumor tissue nucleic acids of mice;[295,296] its incorporation into tobacco mosaic virus nucleic acid is supported by the isolation therefrom of a and b isomers of 8-azaguanylic acid.[297] Other instances of this type of phenomenon are the incorporation of the inhibitors 5-bromouracil into the nucleic acids of *S. faecalis*[298] and 2,6-diaminopurine into the nucleoside phosphates of the mouse.[299]

A new series of analogues in which carbon 2 of the purines is replaced by nitrogen, i.e., the imidazo-1,2,3-triazines, has been developed.[300] A product of the acid degradation of adenine, 4-amino-5-imidazolecarboxamidine[301] is converted upon reaction with nitrous acid into 2-azaadenine (XXXIV);[300] in a similar fashion, 2-azahypoxanthine is obtained from the carboxamide. The adenine analogue exerts a powerful inhibitory effect on a number of microorganisms,[300] and mouse sarcoma 180 cells in tissue culture,[302] and these actions could be blocked by adenine. 2-Azaadenine also inhibits xanthine oxidase.[303]

II. General Properties of Purines and Pyrimidines

Many diverse techniques have been used to study the physical and chemical properties of the purines and pyrimidines and some of these are summarized in this section. Detailed aspects of X-ray, infrared, and ultraviolet studies are considered elsewhere (cf. *Jordan*, Chapter 13; *Beaven, Holiday, and Johnson*, Chapter 14).

1. PHYSICAL PROPERTIES

a. Solubility; Distribution Studies; Chromatography

The pyrimidines and purines which are dealt with here should be classified as *aqueous-soluble* (rather than *organic-soluble*) despite the fact that

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[292] K. Sugiura, G. H. Hitchings, L. F. Cavalieri, and C. C. Stock, *Cancer Research* **10,** 178 (1950).

[293] R. E. F. Matthews, *Nature* **167,** 892 (1951); *J. Gen. Microbiol.* **8,** 277 (1953).

[294] M. R. Heinrich, V. C. Dewey, R. E. Parks, Jr., and G. W. Kidder, *J. Biol. Chem.* **197,** 199 (1952).

[295] J. H. Mitchell, Jr., H. E. Skipper, and L. L. Bennett, Jr., *Cancer Research* **10,** 647 (1950).

[296] L. L. Bennett, Jr., H. E. Skipper, and L. W. Law, *Federation Proc.* **12,** 300 (1953).

[297] R. E. F. Matthews, *Nature* **171,** 1061 (1953).

[298] F. Weygand, A. Wacker, and H. Dellweg, *Z. Naturforsch.* **7b,** 19 (1952).

[299] G. P. Wheeler and H. E. Skipper, *Federation Proc.* **12,** 289 (1953).

[300] D. W. Woolley and E. Shaw, *J. Biol. Chem.* **189,** 401 (1951).

[301] L. F. Cavalieri, J. F. Tinker, and G. B. Brown, *J. Am. Chem. Soc.* **71,** 3973 (1949).

[302] J. J. Biesele, *Cancer* **5,** 787 (1952).

[303] E. Shaw and D. W. Woolley, *J. Biol. Chem.* **194,** 641 (1952).

TABLE I

PHYSICAL PROPERTIES OF PYRIMIDINES AND PURINES

Compound	M.p., °C.	Ref.	Solubility in water: 1 part in	Temp., °C.	Ref.	Distr. coeff.*	R_F†	Ultraviolet spectral properties‡			
								pH	$\lambda_{max.}$, mμ	$\epsilon_{max.} \times 10^{-3}$	Ref.
Pyrimidine	22.5	*a*	very sol.					0.0	242	3.99	*b*
								H_2O	243, 272	2.40, 2.89	*b*
Cytosine	320–25 dec.	*c*	129	25	*c*	0.21	0.47	1–2	210, 276	9.70, 10.0	*d*
picrate‡	300–5 dec.	*c*	1,320	25	*c*			7–10	267	6.13	*d*
								14	282	7.86	*d*
5-Methylcytosine	270 dec.	*e*	222	25	*e*		0.55	1–2	210.5, 283.5	12.0, 9.79	*d*
picrate‡	286 dec.	*e*	1,400	25	*e*			7–10	210.5, 273.5	14.2, 6.23	*d*
								14	289.5	8.05	*d*
5-Hydroxymethyl-cytosine							0.47	1.0	279	9.70	*f*
								7.4	269.5		*f*
								13	283.5		*f*
Uracil	338 dec.	*g*	280	25	*g*	0.40	0.68	1.0	260	7.90	*h*
								4.4–7.2	259.5	8.20	*d*
								12–13	284	6.15	*d*
								14	276.5	6.38	*d*
Thymine	326 dec.	*g*	250	25	*g*	1.11	0.77	1.0	265	7.95	*h*
								4.4–7.2	207, 264.5	9.50, 7.89	*d*
								12–13	291	5.44	*d*
								14	282	5.45	*d*
Orotic acid	345–6 dec.	*i*	550	18	*i*			1–2	205, 280	10.9, 7.52	*d*
								4.4–7.2	207, 278.5	11.6, 7.68	*d*
								12–13	286	5.98	*d*
								14	289	5.35	*d*
Purine	216–17	*j*	2	20	*k*			0.23	260	6.24	*i*
picrate‡	208	*j*						5.94	262.5	8.16	*i*

Adenine	360–5 dec.	m	1,086	Room temp.	m	2.14–2.77	0.36	1.0	262	13.1	n
picrate‡	285–6 dec.	o						6.47	261	12.6	p
Guanine			26,000	40.1	q	0.45	0.25	1.0	252	11.0	n
picrate‡	270–80 dec.	o						5.99	246, 275	10.8, 8.59	p
Isoguanine			16,000	20	k	0.28		6.48	238, 286	7.38, 10.4	p
2,6-Diaminopurine			420	20	k	1.21		6.49	247, 280	7.00, 8.30	p
Hypoxanthine			1,370	23	r	0.54	0.31	1.99	250	10.6	p
								6.44	251	10.3	p
Xanthine			5,500	40.1	q	0.46		2–5	267.5	9.69	s
perchlorate‡	262–4	t						9.71	240, 277.5	8.55, 8.74	s
								14	283	8.88	s
2,8-Dihydroxy-adenine	dec. > 360	r	500,000	23	u			0.0	305	17.4	u
Uric acid			39,480	18	v	0.11		2.3	231, 283	8.51, 11.8	w
								8–8.5	235, 291.5	9.85, 12.4	x
								>12	219.5, 294.5	25.4, 13.5	x

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* Distribution coefficients for system: *n*-butanol; 1 *M* phosphate, pH 6.5; J. F. Tinker and G. B. Brown, *J. Biol. Chem.* **173**, 585 (1948).

† On Whatman No. 1 filter paper in isopropanol (65%, vol./vol.)–water–2 *N* HCl; G. R. Wyatt, *Biochem. J.* **48**, 581, 584 (1951); G. R. Wyatt and S. S. Cohen, *Nature* **170**, 1072 (1952).

‡ The spectral properties refer only to the parent compound and not to the picrate or the perchlorate.

[a] N. Whittaker, *J. Chem. Soc.* **1953**, 1646.

[b] M. P. V. Boarland and J. F. W. McOmie, *J. Chem. Soc.* **1952,** 3716.

[c] W. L. Wheeler and T. B. Johnson, *Am. Chem. J.* **29**, 492 (1903).

[d] D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta* **9**, 199 (1952).

[e] W. L. Wheeler and T. B. Johnson, *Am. Chem. J.* **31**, 591 (1904).

[f] G. R. Wyatt and S. S. Cohen, *Nature* **170**, 1072 (1952).

[g] H. L. Wheeler and H. F. Merriam, *Am. Chem. J.* **29**, 478 (1903).

[h] G. R. Wyatt, *Biochem. J.* **48**, 581, 584 (1951).

[i] M. Bachstez, *Ber.* **63**, 1000 (1930).

[j] E. Fischer, *Ber.* **31**, 2550 (1898); **32**, 435 (1899).

[k] A. Albert and D. J. Brown, *J. Chem. Soc.* **1954,** 2060.

[l] A. Bendich, P. J. Russell, Jr., and J. J. Fox, in press.

[m] H. Bredereck, *in* "Physiologishe Chemie" (Flaschenträger and Lehnartz, eds.), Vol. 1, p. 796. Springer, Berlin, 1951.

[n] H. S. Loring, J. L. Fairley, H. W. Bortner, and H. L. Seagran, *J. Biol. Chem.* **197**, 809 (1952).

[o] J. Meisenheimer, *Z. physiol. Chem.* **114**, 205 (1921).

[p] L. F. Cavalieri, A. Bendich, J. F. Tinker, and G. B. Brown, *J. Am. Chem. Soc.* **70**, 3875 (1948).

[q] J. K. Wood, *J. Chem. Soc.* **83**, 568 (1903).

[r] E. Fischer, *Ber.* **30**, 2226 (1897).

[s] L. F. Cavalieri, J. J. Fox, A. Stone, and N. Chang, *J. Am. Chem. Soc.*, **76**, 1119 (1954).

[t] H. Biltz and A. Beck, *J. prakt. Chem.* [**2**] **118**, 166 (1928).

[u] A. Bendich, G. B. Brown, F. S. Philips, and J. B. Thiersch, *J. Biol. Chem.* **183**, 267 (1950).

[v] W. His, Jr., and T. Paul, *Z. physiol. Chem.* **31**, 1 (1900).

[w] L. F. Cavalieri and A. Bendich, *J. Am. Chem. Soc.* **72**, 2587 (1950).

[x] E. A. Johnson, *Biochem. J.* **51**, 133 (1952).

many of the hydroxy and amino derivatives have a limited water solubilit (Table I). The parent compounds are very soluble in water at ordinar temperatures; the introduction of hydrogen-bonding groups (—OH —NH_2) into pyrimidine or purine results not only in a reduction in wat solubility, but in an increase in melting point as well. (An analogous situation is seen in the pteridine system.[304]) This effect is especially true of th purines: the least soluble is 250,000 times less soluble than purine itsel The replacement of the 2-hydroxyl of xanthine, or of the 6-hydroxyl uric acid, by an amino group is manifested by a further marked decreas in water solubility (cf. Albert[304]). However, all the compounds listed i Table I are easily soluble in dilute aqueous alkali or mineral acid (or both) A common practice in the purification of the less-soluble compounds involves their solution in dilute acid or alkali; crystallization often follow neutralization. Whereas pyrimidine, purine, and the simple alkyl, aryl aryloxy, diarylamino, or halo derivatives are more or less soluble in common organic solvents, the other compounds listed in Table I are, in general extremely insoluble.

For these reasons, preference has been given to the use of aqueous systems for the purpose of separation, purification, and characterization o pyrimidines and purines of biological interest. For example, the countercurrent distribution technique[305] has been successfully applied to suc compounds[306] in a system consisting of *n*-butanol and *M* phosphate, pH 6.5. The coefficients of distribution of pyrimidines and purines betwee the two phases of this system (Table I) are very useful in identification and characterizaticn. The method is also valuable in the separation of mixture of these compounds and for the estimation of homogeneity. Another example is the use of a variety of aqueous systems in column and pape chromatography. These techniques are discussed in detail in Chapters and 7. The R_F values of some pyrimidines and purines in an isopropanol-water-HCl system are listed in Table I.

b. Criteria of Purity and Identity; the Value of Ultraviolet Absorption Spectra Ionization; Tautomerism; the Value of the Isosbestic Point

For discussions of the principles of the purity and identity of organi compounds see Pirie[307] and Eyring.[308]

Table I enumerates the melting and decomposition points of a few pyrimidines and purines, and in some cases those of a salt. The substituted compounds are characterized either by a very high point or by infusibility (the latter is especially true of the hydroxylated purines).[309] This traditional approach (including mixed melting points) can, therefore, only be of limited value in the assessment of purity and identity, and in some instances (see discussion on orotic acid, above) it may even be misleading. However, the number of physical criteria of purity that are available (when taken together) is sufficient to satisfy the most fastidious worker.

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[304] A. Albert, *Quart. Revs. (London)* **6,** 197 (1952).

[305] L. C. Craig, *Fortschr. chem. Forsch.* **1,** 292, 302, 312 (1949).

[306] J. F. Tinker and G. B. Brown *J. Biol. Chem.* **173,** 585 (1948).

[307] N. W. Pirie, *Biol. Revs.* **15,** 377 (1940).

[308] H. Eyring, *Anal. Chem.* **20,** 98 (1948).

When the amount of a specimen to be tested is small, paper chromatography (Chapter 7) is the method of choice. Microgram quantities are used, and the sample, after elution and spectrographic evaluation, is subjected to reanalysis on paper. The specimen, to be judged "pure," must show a single "spot" in more than one solvent system and constant spectral characteristics (at more than one pH) after each chromatographic run. For radioactive materials, coincidence with radioactivity must be established. The same considerations apply to column chromatography (Chapter 6) in that a single band must be demonstrated and the variables are the adsorbents (anion- or cation-exchange resins, starch, etc.) as well as the solvent systems. It is highly unlikely that a mixture of pyrimidines or purines would behave as a homogeneous substance when subjected to all these tests.[310]

When an adequate supply of a preparation is available, and a standard specimen of unquestioned purity is desired, the above techniques as well as the following are used. The substance is subjected to repeated fractionation by crystallization (salt as well as neutral forms), passage through a column, countercurrent distribution, etc., until constancy is achieved with respect to properties such as those listed in Tables I and II, and a corresponding constancy with respect to isotope content if the compound is so labeled. The determination of elementary composition presents no special problem with pyrimidines and purines.

Additional criteria are the reproducibility of ultraviolet data, dissocia-

[309] The eutectic mixtures of a number of pyrimidine and purine derivatives with dicyandiamide exhibit well-defined melting points which are much lower than those of the original derivatives (K. Dimroth and H.-G. Meyer-Brunot, *Biochem. Z.* **323**, 343 (1952)). The determinations are easily carried out on tiny quantities.

[310] It is possible, for example, that the growth response of a bacterial mutant may be due to a small impurity in a purine or pyrimidine preparation. The importance of using homogeneous compounds in biological studies, therefore, cannot be stressed too strongly. Yet, despite the ease and simplicity of paper chromatography, the label on a bottle has often been accepted too literally. As examples of extreme inhomogeneity, the author has examined by paper chromatography a number of commercially available "hypoxanthine" preparations only to find (with one exception) these to contain, on the average, about 20% of adenine. One sample of "guanine" was found to show some four to five "spots," and only one small "spot" corresponded in R_F value to guanine.

tion constant values, and isosbestic points. These are of especial value sinc all three may be determined on microgram quantities from the same set measurements without loss of the sample. These considerations depen upon the fact (Chapter 14) that the nature and magnitude of absorptio of ultraviolet light by a variety of pyrimidines and purines vary with th pH of their aqueous solutions. This is attributable to ionization of one o another group and is derived from the fact that the neutral and ionic specie of a given molecule exhibit different spectra. In order to learn which o these species (or mixture of species) is responsible for a spectrum at particular pH, the dissociation constant must be known. This has involve the prior determination of (apparent) dissociation constant(s), and, from this information,[311-317] spectra have been recorded at a pH so selected tha only the neutral, the anionic, or the cationic form (if any of these is possible) is present in solution.

A variation of this method[318-323] is based upon the observations that the curves relating pH to absorption at particular wavelengths of ultraviolet light are very similar to the dissociation curves. (Such curves, for example for phenol,[318] are superimposable.) In this technique, the spectra are determined over a wide pH range to give a more or less continuous picture of the spectral changes. This often affords a convenient method for estimating apparent dissociation constants and the proportions of absorbing forms present in solution at specific pH values. Because of the difficulty (or, at times, the impossibility) inherent in potentiometric measurements at very low or high pH values, a very feeble dissociation may be missed, and, accordingly, an erroneous conception of ionization behavior is obtained. Such has been the case with the second dissociation of uracil[314] (cf. Shugar and Fox[321]) and xanthine[315] (cf. Cavalieri *et al.*[322]). The second dissociations are demonstrable, however, by spectrophotometry. Apparent dissociation constants should be determined by both methods, and a comparison for certain pyrimidines and purines is given in Table II. It is to be noted that these values are not strictly valid in the thermodynamic sense

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[311] D. J. Brown and L. N. Short, *J. Chem. Soc.* **1953,** 331.

[312] J. R. Marshall and J. Walker, *J. Chem. Soc.* **1951,** 1004.

[313] M. P. V. Boarland, and J. F. W. McOmie, *J. Chem. Soc.* **1952,** 3716.

[314] P. A. Levene, L. W. Bass, and H. S. Simms, *J. Biol. Chem.* **70,** 229 (1926).

[315] A. G. Ogston, *J. Chem. Soc.* **1935,** 1376.

[316] A. Albert, D. J. Brown, and G. Cheeseman, *J. Chem. Soc.* **1951,** 474.

[317] N. Whittaker, *J. Chem. Soc.* **1951,** 1565; **1953,** 1646.

[318] W. Stenström and N. Goldsmith, *J. Phys. Chem.* **30,** 1683 (1926).

[319] J. J. Fox and D. Shugar, *Bull soc. chim. Belges* **61,** 44 (1952).

[320] D. Shugar and J. J. Fox, *Bull soc. chim. Belges* **61,** 293 (1952).

[321] D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta* **9,** 199 (1952).

[322] L. F. Cavalieri, J. J. Fox, A. Stone, and N. Chang, *J. Am. Chem. Soc.* **76,** 1119 (1954).

[323] E. A. Johnson, *Biochem. J.* **51,** 133 (1952).

TABLE II

APPARENT DISSOCIATION CONSTANTS* OF PYRIMIDINES AND PURINES

Compound	Method of measurement						
	Spectrophotometric			Titrimetric			
	pK_{a_1}	pK_{a_2}	Ref.	pK_{a_1}	pK_{a_2}	Concentration	Ref.
Pyrimidine				1.30	—	$M/15$	[a]
Cytosine	4.45	12.2	[b]	4.60	12.16		[c]
5-Methylcytosine	4.6	12.4	[b]				
Uracil	9.5	>13	[b]	9.45	—		[c]
Thymine	9.5	>13	[b]	9.94	—		[c]
1-Methyluracil[d]	9.95	—	[b]	9.99	—	$M/25$	[c]
3-Methyluracil[e]	9.75	—	[b]	9.71	—	$M/25$	[c]
1,3-Dimethyluracil	none		[b]	none			[c]
Orotic acid	~2.8	9.45[f]	[b]	2.40	—		[g]
Barbituric acid	3.9	12.5	[h]	3.98	—		[i]
Barbital	7.85	12.7	[h]	7.91	—		[j]
Purine	2.52	8.90	[k]	2.39	8.93	$M/10$, $M/100$	[l]
Adenine				4.15	9.80		[m]
Guanine				3.3	9.2[n]		[m]
Hypoxanthine				8.8	12.0		[o]
Xanthine	7.53	11.63	[s]	7.7	—	$M/1000$	[p]
Uric acid	5.4	10.6	[t]	5.8	—		[q]
				5.78	(5.85)	$M/10{,}300$	[r]

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[*] Expressed as pK_a, where $pK_a = 1/\log K_a$, and $K_a = (A^-)(H^+)/(AH)$ for "acidic" and $= (B)(H^+)/(BH^+)$ for "basic" dissociations. The designations pK_{a1} and pK_{a2} refer to the first and second dissociations actually measured. Only for pyrimidine (unsubstituted) and purine do the listed pK_{a1} values refer to the basicity of ring nitrogen.

[a] A. Albert, R. Goldacre, and J. Phillips, *J. Chem. Soc.* **1948,** 2240.

[b] D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta* **9,** 199 (1952).

[c] P. A. Levene, L. W. Bass, and H. S. Simms, *J. Biol. Chem.* **70,** 229 (1926)

[d] Referred to as "3-methyluracil" by an alternative nomenclature: D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta* **9,** 199 (1952).

[e] Referred to as "1-methyluracil": see previous reference.

[f] Orotic acid shows $pK_{a3} > 13$. The carboxyl dissociation (pK_{a1}) appears to have a negligible effect on the second and third dissociations since they are very similar to the corresponding values for uracil and thymine.

[g] M. Bachstez, *Ber.* **63,** 1000 (1930).

[h] J. J. Fox and D. Shugar, *Bull. soc. chim. Belges* **61,** 44 (1952).

[i] J. K. Wood, *J. Chem. Soc.* **89,** 1831 (1906); conc. probably $M/64$.

[j] M. E. Krahl, *J. Phys. Chem.* **44,** 449 (1940).

[k] A. Bendich, P. J. Russell, Jr., and J. J. Fox, *J. Am. Chem. Soc.* **76,** (1954), in press.

[l] A. Albert and D. J. Brown, *J. Chem. Soc.* **1954,** 2060.

[m] H. F. W. Taylor, *J. Chem. Soc.* **1948,** 765; conc. from 0.0012 to 0.007 M.

[n] pK_{a2} for guanine = 12.3.

[o] H. F. W. Taylor, "Acid Base Properties of Nucleic Acids," Doctoral Thesis, London Univ., London, 1946; taken from D. O. Jordan, *Progr. Biophys. and Biophys. Chem.* **2,** 51 (1951); *Ann. Rev. Biochem.* **21,** 209 (1952).

[p] A. G. Ogston, *J. Chem. Soc.* **1935,** 1376.

[q] W. His, Jr., and T. Paul, *Z. physiol. Chem.* **31,** 1 (1900).

[r] A. L. Bernoulli and A. Loebenstein, *Helv. Chim. Acta* **23,** 245 (1940); apparently these two separate dissociations ($pK_{a1} = 5.78$ and $pK_{a2} = 5.85$), involving a total of 2 equivalents of alkali per mole of uric acid, escape detection in the spectrophotometric analysis and appear as one ($pK_{a1} = 5.4$).

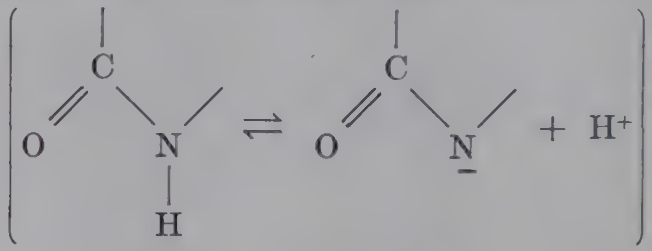
[s] L. F. Cavalieri, J. J. Fox, A. Stone and N. Chang, *J. Am. Chem. Soc.* **76,** 1119 (1954).

[t] E. A. Johnson, *Biochem. J.* **51,** 133 (1952).

(hence only "apparent") since in the spectrophotometric method the
tivity coefficients (which probably do not differ greatly from unity, in
much as the concentrations are between 10^{-5} and 10^{-4} molar) are
known and in the titrimetric technique (concentrations from 10^{-3}
10^{-1} molar) the liquid junction potentials are also not known. Nonet
less, the agreement (Table II) by the two methods is good.

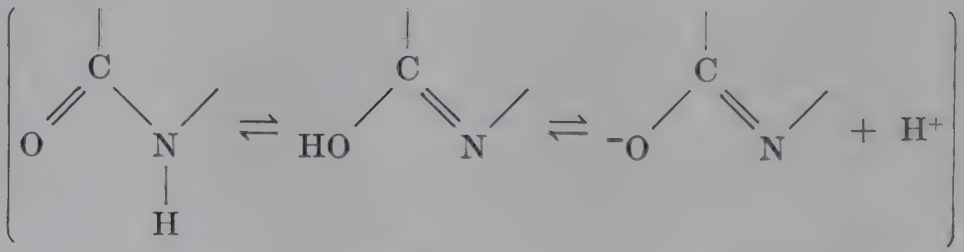
The pK_{a_1} values for the parent compounds pyrimidine and purine ar
measure of the basicity of ring nitrogen and are considerably lower th
those for pyridine (5.23) and imidazole (7.03). (For a discussion of t
basicity of nitrogen-containing heterocycles, see Albert *et al.*[324]) The p
value of purine (8.9) concerns the removal of a proton from the imidaz
portion of the neutral molecule as does the pK_{a_2} (9.80) for adenine. Wh
the pK_{a_1} for adenine (4.15) and guanine (3.3) refer to the amino grou
(—$\overset{+}{N}H_3 \rightleftharpoons$ —$NH_2 + H^+$), it is not yet established which group is involv
in the second and third dissociations for guanine.

As for the derivatives containing potentially tautomeric groups, t
question arises whether, for example, it is the carbonyl form (A)



A A′

or the enol form (B)



A B B′

which dissociates in aqueous solution; i.e., does enolization precede ioniz
tion? Since the ionic forms A′ and B′ are resonance hybrids, they are n
capable of independent existence, and it is not to be expected that ultr
violet spectroscopy can furnish a direct answer. Although the hypothetic
intermediate or uncharged enol (lactim) form may exist in solution, defin
tive evidence for such existence has not been demonstrated by ultraviol
spectroscopy. This might be due to its presence in too small a quantity,
to the possibility that its spectrum might be the same as that for the anion

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[324] A. Albert, R. Goldacre, and J. Phillips, *J. Chem. Soc.* **1948,** 2240.

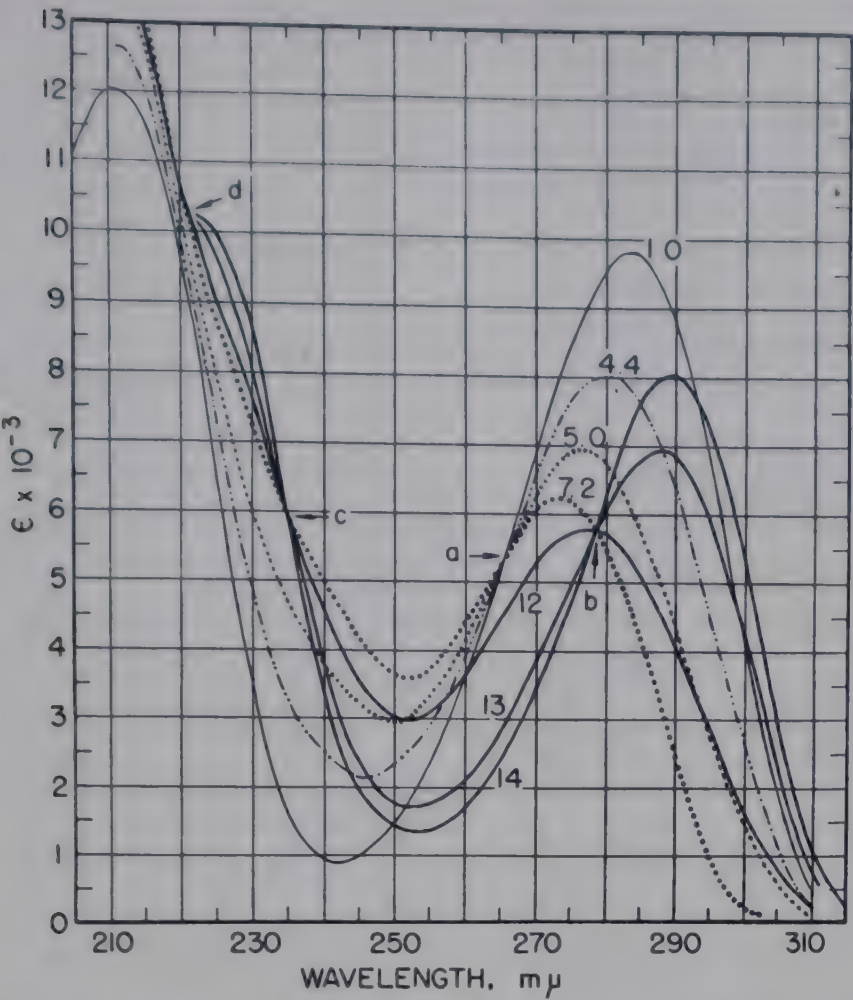

FIG. 1. Ultraviolet absorption spectra of *5-methylcytosine* at pH values (1.0 to 14) indicated. The curve for pH 2, not shown, is identical with that for pH 1.0; those (not shown) for pH 7 to 10 are identical. Isosbestic point *a* is that for pK_{a_1} (= 4.6); *b*, *c*, and *d* are for pK_{a_2} (= 12.4). (Adapted from D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta* **9,** 199 (1952).)

form. Such a derivative would appear to exhibit the phenomenon of "mesomeric tautomerism" which "results in a manifestation of dual character by a compound essentially homogeneous."[325] The existence of individual tautomers (hence a slow tautomeric change) has been inferred[304] from titrimetric data for the related 6-hydroxypteridine and xanthopterin (2-amino-4,6-dihydroxpteridine), but thus far not for pyrimidines and purines.

The principles mentioned above are illustrated for 5-methylcytosine, the spectral changes of which (conc. *ca.* 10^{-4} molar) are shown in Figure 1[321] over the pH range 1 to 14. The curve for pH 1.0 is the same as that for pH 2.0 and shows $\epsilon = 9{,}790$ at wavelength 283.5 mμ. As the pH is increased, this absorption decreases (hypochromic effect) and the position of this maximum shifts towards shorter wavelengths (hypsochromic effect). The values remain constant from pH 7 through 10 ($\epsilon = 6{,}230$ at 273.5 mμ) and hyperchromic and bathochromic effects are observed with further increase in pH until 14 is reached. (The basicity of ring nitrogens, which would be

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[325] L. Hunter, *J. Chem. Soc.* **1945,** 806.

manifested at values below pH 1, has not been measured.) The curves
the cationic form, at pH 1.0, and for the neutral or nondissociated for
at pH 7.2, cross at point *a*, indicating that at this wavelength (266 m
the two forms have the same extinction coefficient. This point is comm
to the curves at all pH values from 1.0 to 7.2, and is called an *isosbestic po*
(point of equal extinction).[326,327] Isosbestic point *a* delineates the equilibri
(pK_{a_1}) between the cationic and neutral forms; isosbestic points *b*, *c*, a
d (pH 7 to 14) are concerned with the equilibrium between the neutral a
anionic forms. Since the neutral form is common to both equilibria,
curve should (and does) pass through all the isosbestic points. The shar
ness of the isosbestic points is one measure of the purity of the absorbi
substance and of the precision in the recording of the spectra. "If curves
a sufficient number of pH values are run, the plot of extinction coefficien
vs. pH at a given wavelength would give a titration curve."[319] From th
the apparent pK_a can be calculated. For homogeneous substances, a p
value calculated from the extinctions at one wavelength should be t
same as that calculated from other wavelengths.

From these considerations, it follows that the nature and even the p
sition(s) of potentially tautomeric groups of an unknown pyrimidine (
purine) derivative may be diagnosed from a study of its spectral behavi
as a function of pH. The molar concentration need not be known since th
spectral behavior is essentially independent of the concentrations usual
employed in ultraviolet spectrophotometry.

Molecular extinction coefficients and absorption maxima are listed
Table I for pyrimidines and purines often encountered in nucleic ac
studies. Whatever differences exist between these and other publishe
values are probably due to small variations in the instruments in use.

2. Chemical Properties

"As the simpler compounds are much less well known than the high
hydroxylated or amino members, a rather distorted impression of pyrim
dine chemistry has grown up, much as if the behavior of benzene we
known only through the reactions of compounds like phloroglucinol."
An analogous statement may be made in regard to purine. Pyrimidine an
purine are probably the most stable members of the series and can surviv
treatment with strong oxidizing (but not reducing) agents, concentrate
sulfuric (100°) and nitric acids.[23,328] The general stability of pyrimidi
may be attributed[328] to the influence of the ring nitrogens which results

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[326] A. Thiel, A. Dassler, and F. Wülfken, *Fortschr. Chem. Physik u. physik. Chem.* 1
(3), 79 (1924).

[327] W. R. Brode, "Chemical Spectroscopy," 2nd ed. p. 249. Wiley, New York, 1943.

[328] B. Lythgoe, *Quart. Revs.* (*London*) **3,** 181 (1949).

an electron deficiency at positions 2, 4, 6, and, to a smaller extent, at the "meta" or 5-position. Hence, pyrimidine exhibits chemical properties that are analogous[328] to those of the more thoroughly studied pyridine system.[329] The stability is altered by the introduction of electron-donating groups such as amino and hydroxyl, and the following sections deal primarily with these derivatives. "Vestiges of the behavior of the parent compound, however, remain; the 2, 4 and 6 position retain their electrophilic character . . .; and substitution by electrophilic reagents is still confined to position 5."[328]

a. Stability Towards Acid and Alkali; Transformations; Nitrous Acid

A practical problem concerning the stability of pyrimidines and purines is encountered in the acid hydrolysis of nucleic acids. The liberation of purines is achieved under much milder conditions than are needed for pyrimidines (Chapters 5–7, 9–11). The usual procedure, *N* acid at 100° for 1 hour, leads, essentially, to a quantitative recovery of the nucleic acid purines as judged by chromatography[330] and differential spectroscopy.[331] However, the isotope dilution technique reveals[332] that this treatment results in a 7 to 8% deamination of adenine and guanine. A considerable hydrolytic conversion of cytosine to uracil occurs during a 90-minute heating (175°) with 10% HCl, but uracil and thymine escape destruction under these circumstances.[330] 5-Methylcytosine is hydrolyzed to thymine by 20% sulfuric acid at 150–160°.[66] No detectable destruction of uracil, cytosine, or thymine is observed upon heating with 98–100% formic acid at 175° for 30–120 minutes;[70,330] the same is true for adenine and guanine during a 30-minutes treatment.[70] A quantitative recovery of adenine, guanine, cytosine, uracil, and thymine is obtained[77] when these compounds are subjected to the action of 12 *N* perchloric acid at 100° for 1 hour, but 5-hydroxymethylcytosine is thus destroyed[54] and a 15% loss of thymine results when the temperature is raised to 110°.[72] (See addendum).

When guanine is refluxed for 32 hours with 25% hydrochloric acid, a 50% yield of xanthine is obtained;[333] a similar treatment of isoguanine for 47 hours also affords about a 50% conversion to xanthine.[179,176] When guanine is heated with 3.4 *N* HCl at 158° for 90 minutes, xanthine (52% yield), ammonia, glycine (53% of the theoretical yield), 4-(or 5-)guanidoimidazole (14%), and a small quantity of glycocyamine are formed. It has been demonstrated with isotopically labeled guanine that the carboxyl, methylene, and amino groups of the glycine arise, respectively, from carbon 4 and 5 and N-7 of the guanine.[301] Upon treatment of guanine with concentrated sulfuric acid at "high temperature," carbons 2 + 6 are liberate as CO_2 and carbon 8 as CO.[334] Both hypoxanthine and adenine yield glycine ammonia, carbon dioxide, and carbon monoxide (the latter probably from the decomposition of formic acid) upon hydrolysis with concentrated HC at 180–200°[335] The amino group of the liberated glycine arises from N- of the adenine;[301] the formation of 4-amino-5-imidazolecarboxamidine from such a hydrolysis was mentioned above. The formic acid resulting from th hydrolysis of adenine with 30% sulfuric acid[336] may be collected by steam distillation.

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[329] H. S. Mosher, *in* "Heterocyclic Compounds" (Elderfield, ed.), Vol. 1, p. 397. Wiley, New York, 1950.

[330] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176,** 715, 703 (1948).

[331] H. S. Loring, J. L. Fairley, H. W. Bortner, and H. L. Seagran, *J. Biol. Chem.* **197,** 809 (1952).

[332] R. Abrams, *Arch. Biochem.* **30,** 44 (1951).

[333] E. Fischer, *Ber.* **43,** 805 (1910).

Data on the action of alkali on pyrimidines and purines are scanty Hypoxanthine is decomposed into ammonia and hydrocyanic acid upon treatment with fused KOH at 200°;[337] adenine and hypoxanthine are, however, unaffected by boiling in aqueous alkali.[335,337] (At 200° in water alone hypoxanthine breaks down to CO_2, NH_3, and formic acid.[337]) Pyrimidine containing hydroxy or amino groups both at position 4 and 5 are unstabl in alkaline solution at room temperature; the instability is markedly increased when, in addition, such a group occupies position 6.[214]

Reference has already been made to the nitrous acid deamination o adenine, guanine, cytosine, and 5-hydroxymethylcytosine to the corresponding hydroxy derivatives. Nitrous acid deaminates both 5-methylcytosine and 5-methylisocytosine to thymine.[70] 2,6-Diaminopurine is deaminated to isoguanine (cf. Davoll[181]) but the latter resists the action o nitrous acid.[179] 8-Aza-adenine and -guanine are converted by nitrous acid to the corresponding hypoxanthine and xanthine analogues.[287] The amino group at C2, 4, and 6 of the pyrimidine moieties of these various compounds therefore do not exhibit the properties expected of typical aromatic amino derivatives, and accordingly are analogous in chemical behavior to the α- and γ-aminopyridines.[329] On the other hand, a behavior approximating that of aromatic amino compounds is shown by 5-aminopyrimidines (cf. Whittaker,[317] Lythgoe,[328] and Rose[338]).

By the action of nitrous acid, a nitroso group can be introduced into the 5-position of certain pyrimidines. This reaction forms the basis of the valuable Traube synthesis of purines (see below). Only those pyrimidines can be nitrosated which contain amino or hydroxy groups in both positions

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[334] W. H. Marsh, *J. Biol. Chem.* **190,** 633 (1951).

[335] M. Krüger, *Z. physiol. Chem.* **16,** 160 (1892); **18,** 351, 423 (1894).

[336] C. D. Stevens, *J. Biol. Chem.* **120,** 751 (1937).

[337] (a) A. Kossel, *Z. physiol. Chem.* **6,** 422 (1882).
(b) **12,** 241 (1888).

[338] F. L. Rose, *J. Chem. Soc.* **1952,** 3448.

4 and 6;[339] this structural requirement is essentially independent of the nature of the substituent at position 2.

When uric acid is heated for 30 hours with acetic anhydride and pyridine, 8-methylxanthine is formed by a ring closure of the intermediate 4,5-diacetylaminouracil.[340] This result was confirmed by Biltz and Schmidt.[341] Carbon dioxide and acetic acid result from the ring closure. Bredereck, *et al.*[340] have recently studied this reaction in detail. Uric acid is converted, upon reaction with formic acid at 220–230°, into xanthine and carbon dioxide.[342]

b. Action of Reducing Agents; Polarographic Behavior

Uracil is reduced to 4,5-dihydrouracil at 75° under 2 atmospheres of hydrogen in the presence of a platinum catalyst.[343] Under identical conditions, cytosine is converted to the same product, with the liberation of ammonia.[344] The resulting dihydrouracil is much less stable to hot acid (as well as alkali at room temperature[314]) and is thereby converted to β-alanine.[343] These reactions have been exploited in a degradation procedure[345] in which each carbon atom of isotopically labeled uracil can be directly analyzed for its isotope content.

Whereas adenine and hypoxanthine are unaffected, aqueous solutions of purine hydrochloride and 2-hydroxypurine each absorb one mole of hydrogen at one atmosphere at room temperature under the influence of a palladium-charcoal catalyst.[346] The resulting dihydropurine derivatives are quite unstable in the presence of dilute mineral acid giving rise to substances containing a diazotizable amino group.

Agents such as sodium amalgam, sodium and ethanol, etc., reduce a number of pyrimidines and effect a rupture of the ring system (cf. Johnson[18] for details). Electrolytic reduction (lead cathode, 7–9°) of 4-methyluracil in 50% sulfuric acid gives 2-hydroxy-4-methyltetrahydropyrimidine and 1,3-diaminobutane.[347] Purone (4,5-dihydro-6-deoxyuric acid) and tetrahydrouric acid result from a similar reduction of uric acid.[348]

Adenine, adenosine, and adenylic acid in 0.1 *N* perchloric acid are reducible at the dropping mercury cathode, whereas guanine, guanosin guanylic acid, cytidine, cytidylic acid, and uracil are not.[349] This was pr posed as a method for the quantitative estimation of as little as one micr gram of adenine (error ± 2%) in a hydrolysate of PNA or DNA. (F theoretical and practical aspects of polarography, see Kolthoff and Li gane.[350]) Pyrimidine, 2-amino-, 6-amino-, 6-hydroxy-, 2,6-diamino-, ar 4,6-diaminopyrimidine are reduced polarographically whereas derivativ such as thymine, isocytosine, and barbituric acid are not.[351] From the polarographic behavior, the reducible group of pyrimidines was found

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[339] B. Lythgoe, A. R. Todd, and A. Topham, *J. Chem. Soc.* **1944,** 315.

[340] H. Bredereck, I. Hennig, and W. Pfleiderer, *Chem. Ber.* **86,** 321, 333 (1953): these investigators attribute the original work to C. F. Boehringer and sons, German Pats. 121,224 and 126,797 (1901).

[341] H. Biltz and W. Schmidt, *Ann.* **431,** 70 (1923).

[342] H. Biltz and A. Beck, *J. prakt. Chem.* [2] **118,** 166 (1928).

[343] E. B. Brown and T. B. Johnson, *J. Am. Chem. Soc.* **45,** 2702 (1923).

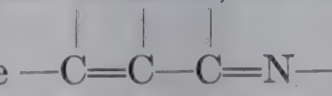
[344] E. B. Brown and T. B. Johnson, *J. Am. Chem. Soc.* **46,** 702 (1924).

[345] V. Lagerkvist, *Acta Chem. Scand.* **7,** 114 (1953).

[346] A. Bendich, P. J. Russell, Jr., and J. J. Fox, *J. Am. Chem. Soc.* **76,** in press (1954).

[347] J. Tafel and A. Weinschenk, *Ber.* **33,** 3378 (1900).

[348] J. Tafel, *Ber.* **34,** 258, 1181 (1901).



involve the $-\overset{|}{C}=\overset{|}{C}-\overset{|}{C}=N-$ system.[351]

Kossel[337b] found that adenine and hypoxanthine (but not guanine caffeine) were decomposed upon heating with zinc and HCl, and that th products (the structures of which were not elucidated) turned a ruby-re when made alkaline. This reductive degradation of adenine (and hypoxar thine) leads to the formation of a diazotizable amine which couples wit *N*-(1-naphthyl)ethylenediamine to form a red-colored dye[352,353] and is en ployed for a colorimetric determination of adenine.[354] Folic acid and AT are reduced by zinc and HCl and produce a red color, but guanine, cytosin isocytosine, thymine, and uracil do not.[352] Hypoxanthine, isoguanine, an xanthine, but not 2,6-diaminopurine or uric acid, are also reduced by zir and HCl at 100°,[355] and it is quite likely that the resulting diazotizab amines are 5-substituted-4-aminoimidazoles.

c. Action of Oxidizing Agents and Ultraviolet Light

As pointed out above, thymine is oxidized by permanganate to urea and cytosine to biuret. The $KMnO_4$ oxidation to urea[345,356] and to oxaluri acid[356] has been utilized for the analysis of the isotope content of specifi carbon atoms of labeled uracil (see Fairley *et al.*[357]). Uracil is also oxidize by ozone in glacial acetic acid, and the products which are obtained ar formylglyoxylurea, oxaluric acid, urea, and oxalic and formic acids.[358] Hydrogen peroxide in the presence of charcoal brings about the oxidation c uracil to isobarbituric, isodialuric, and oxalic acids and urea.[359] When exposed to a solution of ferrous sulfate and sodium bicarbonate in the presence of oxygen, thymine is oxidized and yields urea, acetol, and formic and pyruvic acids.[360] The acetol condenses with *o*-aminobenzaldehyde, yielding the strongly fluorescent 3-oxyquinaldine which turns a deep red upon the addition of ferric chloride, thus furnishing a sensitive color test for thymine. The same oxidation mixture converts cytosine and uracil into dihydroisobarbituric acid, and this compound forms a red complex with ferrous sulfate.[361] Thymine is also oxidized by peroxide in the presence or absence of ferrous sulfate and acetol is obtained upon heating the reaction mixture.[362]

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[349] J. C. Heath, *Nature* **158,** 23 (1946).

[350] I. M. Kolthoff and J. J. Lingane, "Polarography," 2nd ed., Vols. 1 and 2. Intersc ence, New York, 1952.

[351] L. F. Cavalieri and B. A. Lowy, *Arch. Biochem. and Biophys.* **35,** 83 (1952).

[352] A. J. Glazko and L. M. Wolf, *Arch. Biochem.* **21,** 241 (1949).

[353] D. L. Woodhouse, *Arch. Biochem.* **25,** 347 (1950).

[354] H. G. Koritz and F. Skoog, *Arch. Biochem. and Biophys.* **38,** 15 (1952).

[355] S. Friedman and J. S. Gots, *Arch. Biochem. and Biophys.* **39,** 254 (1952).

[356] M. R. Heinrich and D. W. Wilson, *J. Biol. Chem.* **186,** 447 (1950).

[357] J. L. Fairley, L. L. Daus, and B. Krueckel, *J. Am. Chem. Soc.* **75,** 3842 (1953).

[358] T. B. Johnson and R. B. Flint, *J. Am. Chem. Soc.* **53,** 1077 (1931).

The mechanism of this oxidation was investigated by Baudisch and Davidson[363] with the possibility in mind that the intermediate oxidation product might be thymine glycol (XXXV). Thymine was converted quantitatively,[40] upon treatment with bromine water, into 5-bromo-4-hydroxyhydrothymine (XXXVI), and this in turn was transformed[363] into thymine glycol by shaking with moist silver oxide. Acetol (arbitrarily shown in the keto form, XXXVII) and urea were obtained by boiling the glycol in either sodium bicarbonate or barium hydroxide solution and it was concluded[363] that thymine glycol was indeed the intermediate in the peroxide oxidation of thymine. (Thymine can be regenerated from its glycol by hydriodic acid reduction.) Advantage has been taken of this elegant degradation procedure to locate the isotope (C^{14}) in the methyl group of biologically labeled thymine;[364] the resulting acetol was converted by hypoiodite into iodoform. Acetol is also obtained when 5-methylcytosine is subjected[365] to the Baudisch-Davidson procedure.

Uracil, cytosine, and thymine absorb iodine in aqueous bicarbonate solution; urea is formed when the reaction mixtures are heated, and, in the case of thymine, acetol is also formed.[366] Uracil, xanthine, and guanine each absorb four equivalents of iodine from an alkaline solution,[367,368] whereas uric acid absorbs only one mole[367] and adenine does not react; although the chemistry of these reactions has not yet been clarified, they serve as analytical procedures. [Cf. *Dische*, Chapter 9.]

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[359] C. R. Schwob and L. R. Cerecedo, *Proc. Am. Soc. Biol. Chemists, J. Biol. Chem.* **105,** lxxvi (1934).

[360] T. B. Johnson and O. Baudisch, *J. Am. Chem. Soc.* **43,** 2670 (1921).

[361] O. Baudisch, *J. Biol. Chem.* **60,** 155 (1924).

[362] O. Baudisch and L. W. Bass, *J. Am. Chem. Soc.* **46,** 184 (1924).

[363] O. Baudisch and D. Davidson, *J. Biol. Chem.* **64,** 233 (1925).

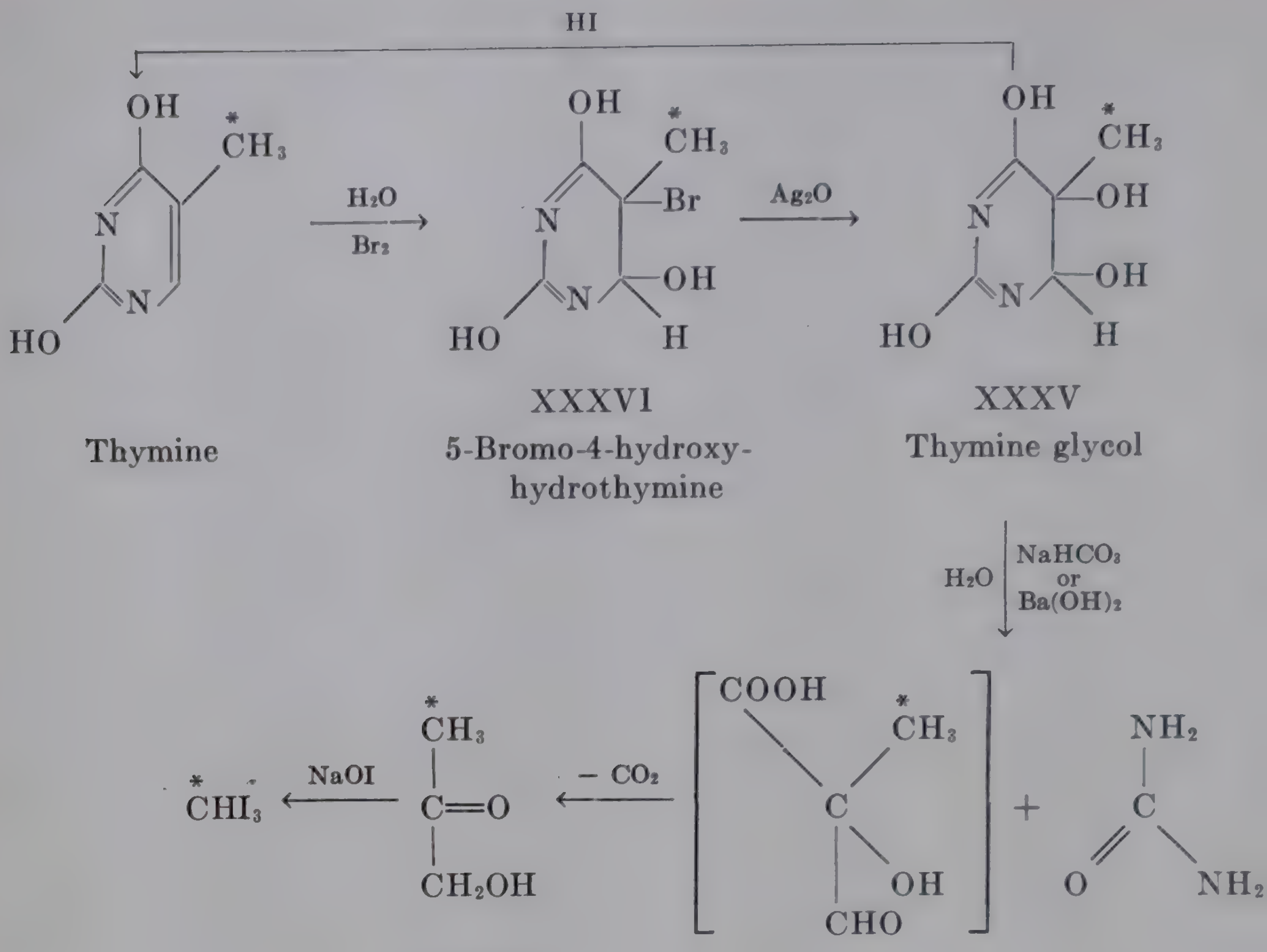
[364] D. Elwyn and D. B. Sprinson, *J. Am. Chem. Soc.* **72,** 3317 (1950).

[365] H. H. Harkins and T. B. Johnson, *J. Am. Chem. Soc.* **51,** 1237 (1929).

[366] L. W. Bass and O. Baudisch, *J. Am. Chem. Soc.* **46,** 181 (1924).

[367] M. Z. Grynberg, *Biochem. Z.* **253,** 143 (1932).

[368] W. Klein, *Z. physiol. Chem.* **231,** 125 (1935).



Thymine

XXXVI
5-Bromo-4-hydroxy-hydrothymine

XXXV
Thymine glycol

XXXVII
Acetol

Iodoform

Urea

The chloric acid oxidation of guanine[35] to guanidine (and parabanic acid) described above, has been utilized in isotope studies to isolate and analyze carbon #2.[271,369,370] Guanidine (from C-2) is also derived from an acid-permanganate oxidation of guanine;[356] urea (mainly from C-8) and carbon dioxide are formed as well (cf. Edmonds *et al.*[371]). The acid-permanganate oxidation of triply labeled adenine produces urea which arises from the 1,7- and 3,9-nitrogen atoms.[301]

The oxidative degradations of uric acid to alloxan, murexide, allantoin, etc., are well known and are ably discussed in detail elsewhere.[13,18,163] These degradations have been applied to biological studies involving isotopes[372-376] and to isotopically labeled uric acid.[377] Uric acid in alkaline solution is oxi-

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[369] A. A. Plentl and R. Schoenheimer, *J. Biol. Chem.* **153,** 203 (1944).

[370] G. B. Brown, P. M. Roll, A. A. Plentl, and L. F. Cavalieri, *J. Biol. Chem.* **172,** 469 (1948).

[371] M. Edmonds, A. M. Delluva, and D. W. Wilson, *J. Biol. Chem.* **197,** 251 (1952).

[372] J. M. Buchanan, J. C. Sonne, and A. M. Delluva, *J. Biol. Chem.* **173,** 81 (1948).

[373] J. M. Buchanan, *J. Cellular Comp. Physiol.* **38,** Suppl. 1, 143 (1951).

[374] D. Shemin and D. Rittenberg, *J. Biol. Chem.* **167,** 875 (1947).

[375] J. L. Karlsson and H. A. Barker, *J. Biol. Chem.* **177,** 597 (1949).

[376] D. Elwyn and D. B. Sprinson, *J. Biol. Chem.* **184,** 465 (1950).

[377] L. F. Cavalieri and G. B. Brown, *J. Am. Chem. Soc.* **70,** 1242 (1948).

dized by oxygen in the presence of catalytic amounts of copper;[378] the spectral changes accompanying this oxidation are identical[378] with those that occur when uric acid is oxidized by uricase (cf. Bentley and Neuberger.[379]).

In addition to the alterations brought about by the oxidizing agents discussed above, certain pyrimidines and purines undergo profound, but poorly understood, changes when their solutions are exposed to ultraviolet light. For example, thymine,[380] uracil,[338] isoguanine,[381] and adenylic and uric acids[382] show extensive loss of their ultraviolet absorption spectra after such exposure. In the case of thymine,[380] the pyrimidine ring is disrupted with the formation of urea and pyruvic acid. When uracil and uridine solutions are irradiated in the 230–280 $m\mu$ region, the loss in absorption is largely restored by acidification of the irradiated solutions.[383]

d. *Color Tests*

Although they have been largely superseded by the modern and more specific chromatographic, spectrophotometric, and polarographic techniques, color reactions are often employed to follow the course of a chemical reaction, the effectiveness of a fractionation or isolation procedure, or for the identification and quantitative estimation of pyrimidines and purines. Reference has already been made in the previous three sections to a number of color tests. The Wheeler-Johnson test[384] for uracil and cytosine depends upon the conversion of these compounds to 5,5-dibromo-4-hydroxyhydrouracil, the bromine atoms of which are replaced by treatment with barium hydroxide solution to give isodialuric and dialuric (2,4,5,6-tetrahydroxypyrimidine) acids. The barium salts of these acids are purple. The test is not given by thymine, 5-methylcytosine, or pyrimidine nucleosides.[14] In an adaptation of this reaction,[385] uracil, cytosine, isocytosine, 5-bromocytosine, and thiouracil are also converted with bromine water to the dibromohydroxyhydrouracil; the latter reduces the uric acid reagent, lithium arsenotungstate, to produce a blue color which is measured quantitatively. Uracil, cytosine, and thymine, but not N-3-substituted pyrimidines, couple with the Pauly reagent, diazobenzenesulfonic acid, to give a red diazo dye following alkalinization[14,386,387] (cf. Lythgoe *et al.*[339]). This reaction serves as the basis of a microestimation technique for thymine.[388-390]

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[378] M. Griffiths, *J. Biol. Chem.* **197,** 399 (1952).

[379] R. Bentley and A. Neuberger, *Biochem. J.* **52,** 694 (1952).

[380] L. W. Bass, *J. Am. Chem. Soc.* **46,** 190 (1924).

[381] M. M. Stimson, *J. Am. Chem. Soc.* **64,** 1604 (1942).

[382] D. Rapport and A. Canzanelli, *Science* **112,** 469 (1950).

[383] R. L. Sinsheimer and R. Hastings, *Science* **110,** 525 (1949).

[384] H. L. Wheeler and T. B. Johnson, *J. Biol. Chem.* **3,** 183 (1907).

[385] M. Soodak, A. Pircio, and L. R. Cerecedo, *J. Biol. Chem.* **181,** 713 (1949).

[386] T. B. Johnson and S. H. Clapp, *J. Biol. Chem.* **5,** 163 (1908).

Guanine and xanthine, but not adenine or hypoxanthine, react with th Folin phenol reagent to give a blue color in an analytical procedure.[391]

e. Salt Formation

The purines and pyrimidines form a variety of salts with many acid and metal ions, a detailed list of which has been compiled by Levene an Bass.[13] Many investigators have, since 1858,[35] employed ammoniacal silve nitrate to precipitate, isolate, and estimate purines as sparingly solubl silver salts.[27,392-394] Silver salts of the purines may also be formed quantitatively in dilute sulfuric acid solution[331,395] and the purines are easily regenerated upon treatment with hydrochloric acid. The pyrimidines o nucleic acid origin do not precipitate as silver salts under acid conditions, but do so in alkali.[13] Poorly soluble cuprous-purine complexes are formed when purines are boiled in the presence of cupric sulfate and sodium bisulfite[335,396a] or cuprous oxide.[397] The purines can be recovered following reaction with hydrogen sulfide. The common purines and pyrimidines form relatively insoluble complexes with mercury[330] when treated with mercuric acetate solution at pH 6.2. Silver salts and chloromercuric derivatives of the purines[335,393] condense with acetohalo-sugars to form purine nucleosides.[398,399]

The picrates of many purines and pyrimidines are easily crystallizable, well-defined salts and are valuable for purposes of isolation, characterization, and quantitative estimation. Properties of certain picrates are given in Table II. Picrate formation is the basis of a quantitative procedure for adenine and guanine[393,396b] The crystalline argentipicrate is used in a determination of hypoxanthine.[400] The presence of a high-intensity band for picric acid in the spectral region 350–400 mμ and the absence of signficant light absorption for many purines and pyrimidines in this region lend themselves to a simple micromethod for the determination of the molecular

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[387] T. B. Johnson and J. H. Derby, *Am. Chem. J.* **40,** 444 (1908).

[388] G. Hunter, *Biochem. J.* **30,** 745 (1936).

[389] D. L. Woodhouse, *Biochem. J.* **44,** 185 (1949).

[390] E. D. Day and W. A. Mosher, *J. Biol. Chem.* **197,** 227 (1952).

[391] G. H. Hitchings, *J. Biol. Chem.* **139,** 843 (1941).

[392] Z. Neubauer, *Z. anal. Chem.* **6,** 33 (1867).

[393] G. Bruhns, *Ber.* **23,** 225 (1890); *Z. physiol. Chem.* **14,** 533 (1890).

[394] E. Salkowski, *Arch. ges. Physiol.* **69,** 268 (1897–98).

[395] S. E. Kerr, K. Seraidarian, and M. Wargon, *J. Biol. Chem.* **181,** 761 (1949).

[396] G. H. Hitchings and C. H. Fiske, *J. Biol. Chem.* (a) **140,** 491 (1941); (b) **141,** 827 (1941).

[397] S. Graff and A. Maculla, *J. Biol. Chem.* **110,** 71 (1935).

[398] E. Fischer and B. Helferich, *Ber.* **47,** 210 (1914).

[399] J. Davoll and B. A. Lowy, *J. Am. Chem. Soc.* **73,** 1650 (1951).

[400] G. H. Hitchings, *J. Biol. Chem.* **143,** 43 (1942).

weights of picrates. This method[401] was used to establish the molecular weight of the aglycone (adenine) of cordycepin.[135,136] Purines and pyrimidines can be recovered from their picrates by extracting hydrochloric acid solutions of these salts with benzene or ether,[370,402] by the use of anion-exchange resins (cf. Davoll and Lowy[399]) or by saturating dry ether or acetone suspensions with HCl gas; in the last procedure, the purines and pyrimidines are often obtained as the crystalline hydrochlorides.

III. Synthetic Methods

It is a tribute to the ingenuity of the earlier workers that many of the recent syntheses of pyrimidines and purines employing isotopes have been fashioned from methods (see General References) devised by the earlier workers. There have been a few newer developments, and emphasis shall be given to these in the following sections.

1. GENERAL METHODS FOR PYRIMIDINES AND FOR THE INTRODUCTION OF ISOTOPES

Since they are cyclic amidines, pyrimidines have been synthesized by condensing amidines, or substituted amidines such as ureas, thioureas, and guanidines, with the appropriate compound containing at least three carbon atoms in a chain. The latter include esters of malonic, acetoacetic, cyanoacetic, and their substituted acids, or the free acids, β-diketones, α, β-unsaturated esters, malononitrile, 1,3-dialdehydes, etc. Examples of this type of synthesis were given for the preparation of thymine, uracil, and orotic acid and have been adapted for the introduction of isotopes.[235,369] Depending upon the choice of reactants, pyrimidines with varying degrees of substitution may be obtained. For instance, guanidine and nitromalonaldehyde condense in mildly alkaline solution to give 2-amino-5-nitropyrimidine which is hydrolyzed to 2-hydroxy-5-nitropyrimidine on boiling with aqueous ammonia; the latter compound is prepared also from urea and nitromalonaldehyde.[403] 2-Aminopyrimidine is formed by reacting guanidine and β-ethoxyacrolein acetal in ethanolic HCl.[404] Convenient syntheses of uracil and isocytosine are afforded by the condensation, respectively, of urea and guanidine with formylacetic acid (formed *in situ* from malic acid) in fuming sulfuric acid.[405,406] Similarly, thymine and 5-methylisocytosine are derived from β-methylmalic acid.[407]

[401] K. G. Cunningham, W. Dawson, and F. S. Spring, *J. Chem. Soc.* **1951,** 2305.

[402] J. K. Parnas, *Biochem. Z.* **206,** 16 (1929).

[403] W. J. Hale and H. C. Brill, *J. Am. Chem. Soc.* **34,** 82 (1912).

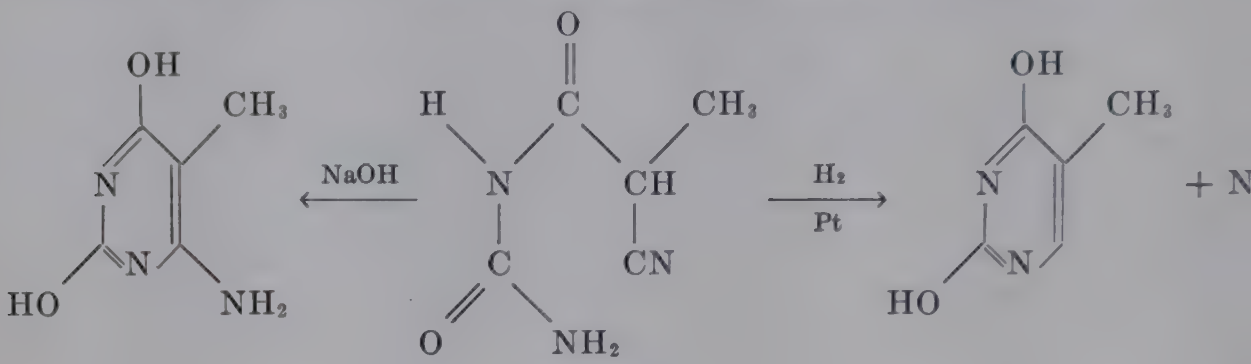
[404] R. W. Price and A. Moos, *J. Am. Chem. Soc.* **67,** 207 (1945).

[405] D. Davidson and O. Baudisch, *J. Am. Chem. Soc.* **48,** 2379 (1926).

[406] W. T. Caldwell and H. B. Kime, *J. Am. Chem. Soc.* **62,** 2365 (1940).

[407] H. W. Scherp, *J. Am. Chem. Soc.* **68,** 912 (1946).

An interesting synthesis of thymine, shown below, was developed Bergmann and Johnson.[408] Urea and methylcyanoacetic acid are hea with acetic anhydride to give methylcyanoacetylurea (XXXVIII), whi as expected, rearranges under the influence of aqueous alkali to yield aminothymine. However, XXXVIII absorbs one mole of hydrogen in t presence of a platinum catalyst to give ammonia and thymine:



XXXVIII

4-Aminothymine Methylcyanoacetylurea Thymine

This reaction is based upon a similar conversion of cyanoacetylurea uracil and ammonia[409] under reducing conditions (H_2 and a nickel catalys It was believed[409] that the cyano group was first reduced to an imino grou which in turn was hydrolyzed to ammonia and an aldehydo group prior cyclization. Although this explanation is plausible, it does not receive su port from the catalytic reduction of 4-aminouracil to uracil.[410] Cyanoacet urea is prepared by the action of phosphorus oxychloride upon urea a cyanoacetic acid[36] and undergoes a base-catalyzed ring closure to 4-amin uracil. The latter is also made by refluxing urea and cyanoacetic ester ethanol containing sodium ethoxide.[411]

Urea (N^{15}) and cyanoacetal [$(C_2H_5O)_2CHCH_2CN$] condense in boili butanol in the presence of sodium butoxide to give an intermediate urei which cyclizes to 1,3-labeled cytosine upon acidification.[412] In a simil manner, guanidine (N^{15}) affords isotopically labeled 2,4(or 6)-diamin pyrimidine.[413] Other examples of this type of synthesis are given below the section on purines.

a. *Transformations; the Value of Halogen and Mercapto Derivatives*

Because certain pyrimidines are difficult to prepare by more direct mea a number of transformations of more easily accessible compounds ha

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[408] W. Bergmann and T. B. Johnson, *J. Am. Chem. Soc.* **55,** 1733 (1933).

[409] H. Rupe, A. Metzger, and H. Vogler, *Helv. Chim. Acta* **8,** 848 (1925).

[410] J. C. Ambelang and T. B. Johnson, *J. Am. Chem. Soc.* **63,** 1934 (1941).

[411] M. Conrad, *Ann.* **340,** 310 (1905).

[412] A. Bendich, H. Getler, and G. B. Brown, *J. Biol. Chem.* **177,** 565 (1949).

[413] A. Bendich, W. D. Geren, and G. B. Brown, *J. Biol. Chem.* **185,** 435 (1950).

een developed, and some of these have been mentioned above. A wide
riety of 2,4- and 6-hydroxypyrimidines including uracil, 5-nitro-, and
methyluracil, barbituric acid, 6-amino-4-hydroxy-2-methylthiopyrimi-
ne, etc. are converted by means of $POCl_3$ into the corresponding chloro
erivatives. (A considerable improvement in the chlorination procedure
volves the addition of dimethylaniline to the reaction mixtures.[289,312,317,414])
ny or all of the chloro atoms of these compounds are often readily replaced
hydrogen or by amino, mercapto, alkoxy groups, etc. (cf. Johnson and
ahn[14]). For example, 2,6(or 4)-dichloropyrimidine and benzenesulfon-
ydrazide condense smoothly and the resulting hydrazino derivative is
onverted to pyrimidine upon alkaline hydrolysis.[415] Dechlorination may
so be effected by catalytic hydrogenation, or reduction with phosphonium
dide or zinc and water, thus making available many new pyrimidines.[14,113,
3,312,317,415]

Amination of chloropyrimidines usually proceeds smoothly, and, in the
ses of 5-nitro-2,6-dichloropyrimidine and its 4-methyl homologue, re-
acement of only the 6-chloro atom by an amino group may be
fected.[113,286] However, amination of other 2,6(or 4)-dichloro- or -diethoxy-
yrimidines leads to mixtures of monoamino isomers[14] and a method utiliz-
g mercapto derivatives has been developed which obviates this difficulty.
The mercapto derivatives have been prepared by total synthesis involving
iourea or substituted thioureas in the type of condensation discussed
ove, and through the replacement of chloro atoms by alkali hydrosul-
des.[14,47,245] A new method, bypassing the halogenated pyrimidines, de-
ends upon the direct thiation of uracils by heating with phosphorus penta-
lfide in an inert medium such as xylene or tetralin.[416-418] The resulting
,6-pyrimidinedithiols usually lead to 6-amino-2-pyrimidinethiols exclu-
vely upon reaction with ammonia or amines.[417] Thus, the dithiol com-
ounds derived from uracil and thymine give excellent yields of 2-cytosine-
iol and 5-methyl-2-cytosine-thiol, respectively.[419,418] These compounds,
turn, are converted to cytosine and 5-methylcytosine[419,418] by an appli-
tion of the Wheeler-Liddle desulfurization technique.[245] The reactions are
lustrated:

J. Baddiley and A. Topham, *J. Chem. Soc.* **1944,** 678.

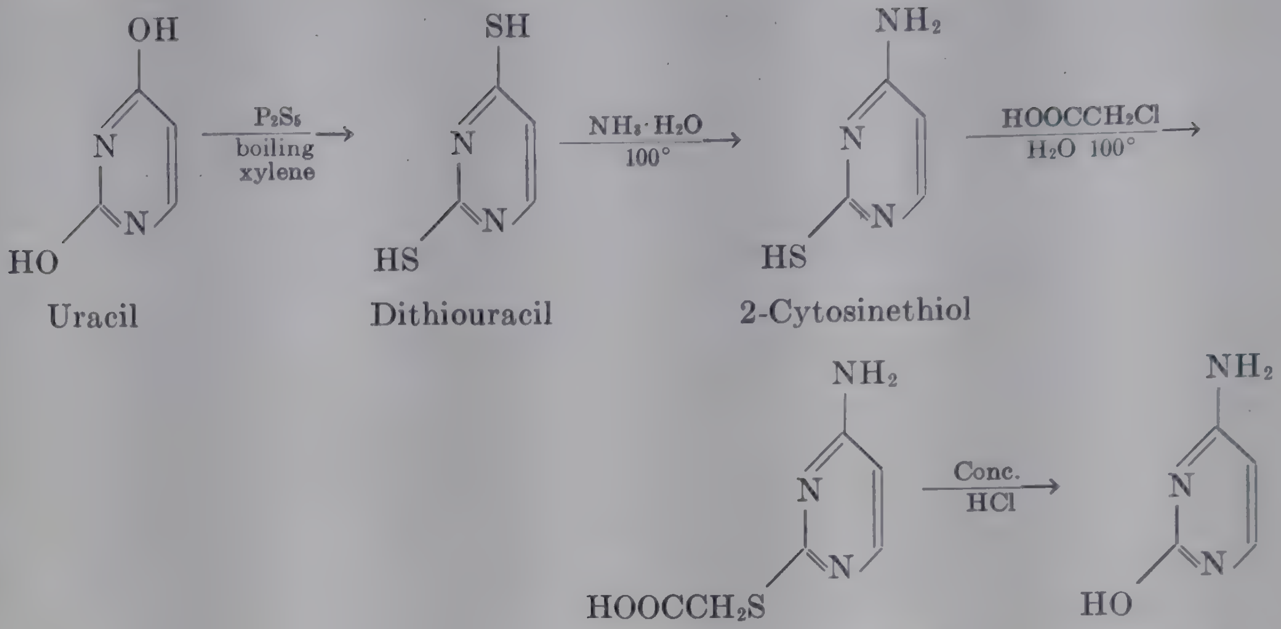
M. P. V. Boarland, J. F. W. McOmie, and R. N. Timms. *J. Chem. Soc.* **1952,** 4691.

G. B. Elion and G. H. Hitchings, *J. Am. Chem. Soc.* **69,** 2138 (1947).

P. B. Russell, G. B. Elion, E. A. Falco, and G. H. Hitchings, *J. Am. Chem. Soc.* **71,** 2279 (1949).

D. J. Brown, *J. Soc. Chem. Ind.* (*London*) **69,** 353 (1950).

G. H. Hitchings, G. B. Elion, E. A. Falco, and P. B. Russell, *J. Biol. Chem.* **177,** 357 (1949).



Uracil Dithiouracil 2-Cytosinethiol

2-Carboxymethylthiocytosine Cytosine

The carboxymethylthiopyrimidines easily form from chloroacetic acid and mercaptopyrimidines and exhibit an extreme range of stability towards acid.[182,245,420] Convenient syntheses of 2-C^{14}-labeled uracil and thymine[421] exploit the ease with which the Wheeler-Liddle desulfurization proceeds with the 2-thio derivatives.

An unwanted mercapto group may be replaced by a hydrogen atom by treatment with Raney nickel. 4,5-Diamino-6-hydroxypyrimidine was obtained from its 2-mercapto derivative in the first application of this technique to pyrimidines.[287] Other examples of compounds prepared by reductive desulfurization with Raney nickel are 4(or 6)-amino- and 4(or 6)-hydroxypyrimidine,[422,418] 4,5-diaminopyrimidine,[423] 4-hydroxy-6-methylpyrimidine,[312] and pyrimidine.[415]

Another method for the synthesis of mercapto derivatives proceeds from the interaction of a halo derivative with thiourea in boiling ethanol solution. 2-Mercapto-, 4-mercapto-, and dithiouracil are obtained directly from the corresponding chloropyrimidines without isolation of the intermediate thiouronium salts.[424] The thiouronium salt from 2-chloro-4,6-dimethylpyrimidine and thiourea can be isolated, and this yields the 2-mercapto derivative upon alkaline hydrolysis. Thiourea transforms 2,5-dichloro- into 2-mercapto-5-chloro-pyrimidine.[424] This reaction is an example of the relative stability of halogen atoms at C-5 of pyrimidines.[14,214,425]

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[420] G. H. Hitchings and P. B. Russell, *J. Chem. Soc.* **1949,** 2454.

[421] L. L. Bennett, *J. Am. Chem. Soc.* **74,** 2432 (1952).

[422] L. F. Cavalieri and A. Bendich, *J. Am. Chem. Soc.* **72,** 2587 (1950).

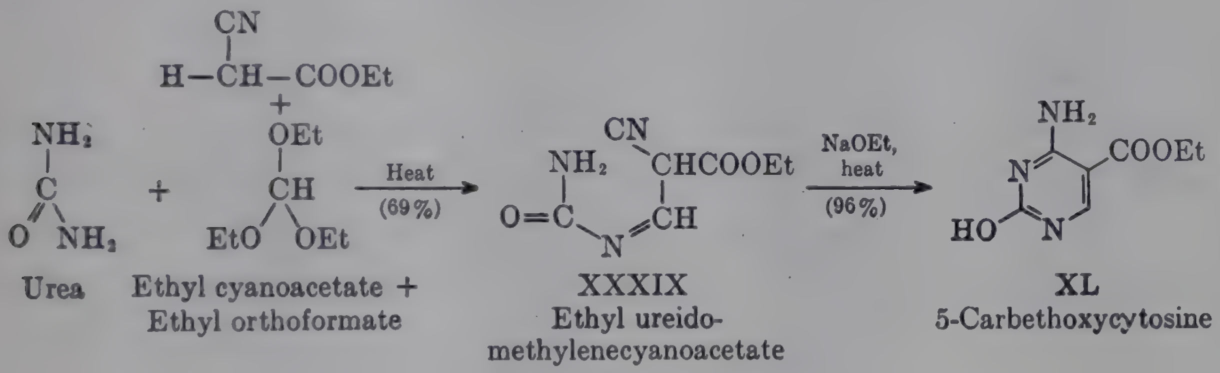
[423] D. J. Brown, *J. Appl. Chem. (London)* **2,** 239 (1952).

[424] J. F. W. McOmie and M. P. V. Boarland, *Chemistry & Industry* **1950,** 602; *J. Chem. Soc.* **1951,** 1218; **1952,** 3722.

[425] M. Yanai, *J. Pharm. Soc. Japan* **62,** 95 (1942).

b. Newer Methods

A convenient method for the preparation of a variety of pyrimidines has been developed by Whitehead.[426] Urea, ethyl orthoformate, and ethyl cyanoacetate condense when heated together to give ethyl ureidomethylenecyanoacetate (XXXIX), in 69 % yield, and this in turn, by refluxing in ethanol-sodium ethoxide, isomerizes quantitatively to 5-carbethoxycytosine (XL). (Cf. Wheeler and Johns[427] for a synthesis of XL by an indirect method.) If ethyl malonate is used instead, diethyl ureidomethylenemalonate (XLI) is formed; it can also be obtained by permitting urea and ethoxymethylenemalonic ester to react at room temperature in ethanol-sodium ethoxide, but the yield is smaller.[426] XLI cyclizes to 5-carbethoxyuracil (XLII).

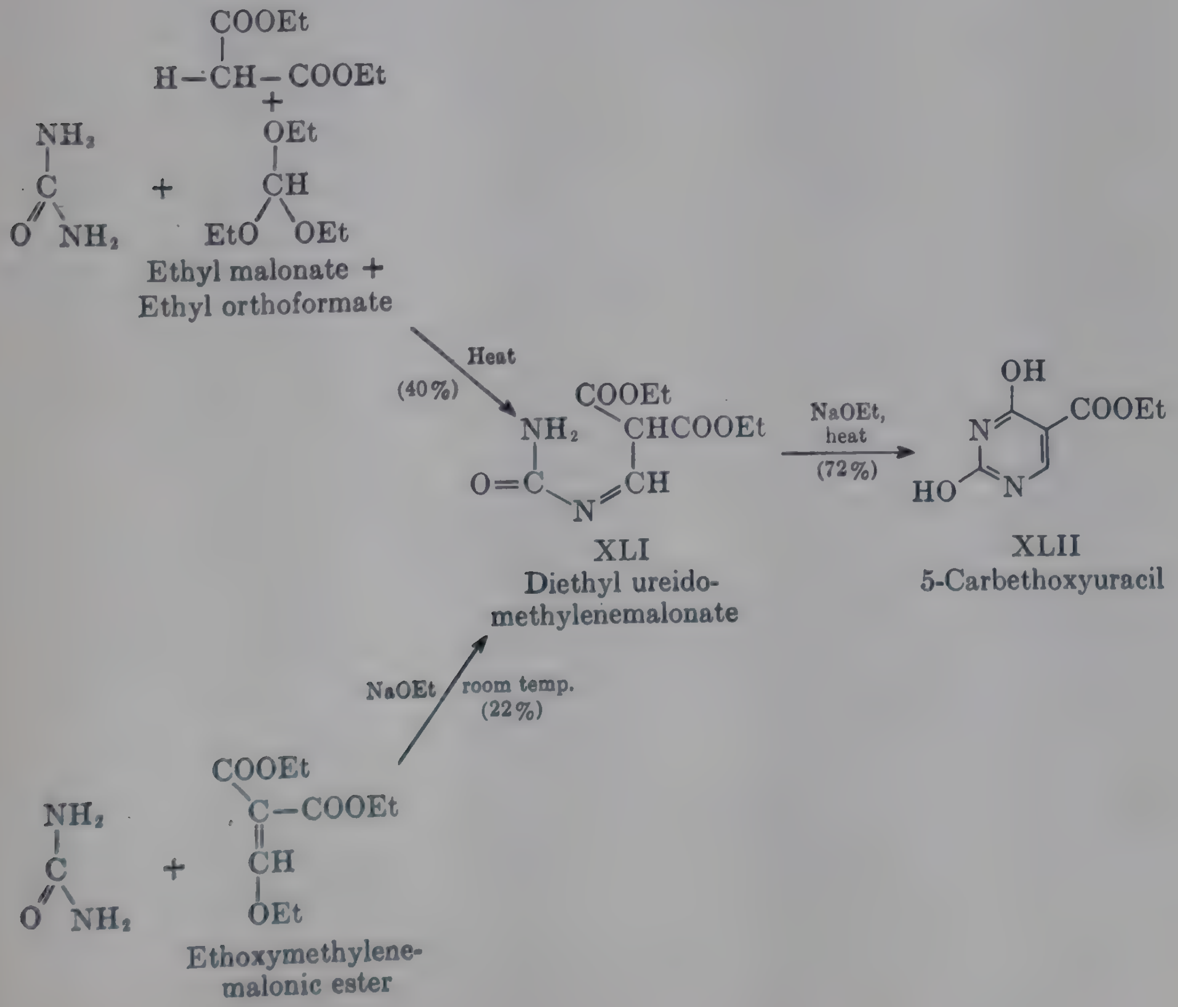


Urea

Ethyl cyanoacetate +
Ethyl orthoformate

XXXIX
Ethyl ureidomethylenecyanoacetate

XL
5-Carbethoxycytosine



Ethyl malonate +
Ethyl orthoformate

XLI
Diethyl ureidomethylenemalonate

XLII
5-Carbethoxyuracil

Ethoxymethylenemalonic ester

N-substituted ureas and other active methylene compounds (malononitrile, acetoacetate, etc.) may also be used in this method. The application of the newer reducing agents, lithium aluminium and boron hydrides, to the 5-carbethoxy or 5-carboxy compounds might prove useful in the preparation of 5-hydroxymethylpyrimidines (cf. Wyatt and Cohen[54,80]). In connection with the free acid forms of XL and XLII, they are both transformed into uracil[226,427] by heating with 20% sulfuric acid at 160–170°, yet uracil-4-carboxylic acid (orotic acid) is not decarboxylated at 200°.[227]

A new reaction of acetylene with nitriles leads to the synthesis of 2,4-disubstituted pyrimidines.[428] For example, 2,4-dimethylpyrimidine results when acetonitrile, potassium, and acetylene are autoclaved at 175–200°.

2. General Methods for Purines and for the Introduction of Isotopes

a. From Purines

The syntheses and transformations in the purine series developed by Fischer,[9] some of which were discussed above, were based mainly upon 2,6,8-trichloropurine. The preparation of this valuable intermediate has been improved considerably by using dimethylaniline in the reaction between uric acid and $POCl_3$.[429] When a limited amount of triethylamine is used in place of the dimethylaniline, 8-chloroxanthine is obtained from monopotassium urate, and with an excess of triethylamine, 2,8-dichloro-6-diethylaminopurine is formed.[430] The latter reaction may be of a general nature since the hydroxyls of hypoxanthine and xanthine are all replaced by diethylamino groups upon refluxing with triethylamine and $POCl_3$. The resulting 6-diethylaminopurine is also prepared from 6-methylmercaptopurine by heating with aqueous diethylamine in a sealed tube.[140]

Other 6-aminopurines are formed smoothly upon reacting 6-methylmercaptopurine with the appropriate amines, but, unlike the mercaptopyrimidines, 6-mercaptopurine does not react satisfactorily. As with pyrimidines,[416-418] 6-mercaptopurine is prepared by heating hypoxanthine with P_2S_5 in tetralin.[140] Another synthesis that has a counterpart in the pyrimidine series[424] is the direct formation of 6-mercaptopurine from 6-chloropurine by boiling with alcoholic thiourea.[346] 6-Chloropurine (formed by reacting hypoxanthine with $POCl_3$ in the presence of dimethylaniline) and 6-mercaptopurine are reduced to purine, respectively, upon catalytic hy-

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[426] C. W. Whitehead, *J. Am. Chem. Soc.* **74,** 4267 (1952); **75,** 671 (1953).

[427] H. L. Wheeler and C. O. Johns, *Am. Chem. J.* **38,** 594 (1907).

[428] T. L. Cairns, J. C. Sauer, and W. K. Wilkinson, *J. Am. Chem. Soc.* **74,** 3989 (1952).

[429] J. Davoll and B. A. Lowy, *J. Am. Chem. Soc.* **73,** 2936 (1951).

[430] R. K. Robins and B. E. Christensen, *J. Am. Chem. Soc.* **74,** 3624 (1952).

drogenation and Raney nickel desulfurization.[346] The mercapto group of 2-mercaptohypoxanthine was replaced by hydrogen via nitric acid oxidation in an early synthesis of hypoxanthine.[30] Syntheses of adenine may be effected by the nitric acid-hydrogen peroxide oxidation of 2-mercaptoadenine[30] or by desulfurization with Raney nickel.[182]

Other transformations have been utilized in partial syntheses of isotopically labeled purines. 1,3-Labeled xanthine[369,431] and hypoxanthine[431] are obtained from the nitrous acid deamination, respectively, of N^{15}-guanine and adenine. Stably-bound deuterium or tritium atoms are introduced into the adenine and guanine molecules by isotope interchange from heavy water in the presence of a platinum catalyst.[432] The isotopes undoubtedly enter at position 8 of guanine and positions 2 or 8 (or both) of adenine, and the amount of exchange is a function of the isotope content of the aqueous media used.

An amino group may be introduced into the 8-position of purines unsubstituted at C-8 by coupling with 2,4-dichlorobenzenediazonium chloride and reducing the resulting diazo compound with sodium hydrosulfite.[433,422] For other transformations including methylations on ring nitrogen, see Fischer,[9] Johnson,[18] and Biltz.[163]

b. From Pyrimidines

The most versatile and widely used method for the synthesis of purines was developed in 1900 by Traube.[36] This method may be considered to consist of two parts: (*a*) the preparation of the appropriate 4,5-diaminopyrimidine, and (*b*) ring closure to the purine. Traube introduced the amino group into the 5-position of 4-amino-6-hydroxy- and 4,6-diaminopyrimidines (bearing a mercapto, hydroxyl, or amino group at C-2) by nitrosation (cf. Lythgoe *et al.*[339]) followed by ammonium sulfide reduction. Variations of this method include reduction of the nitroso group with hydrosulfite,[182,434,435] the use of 5-nitro derivatives and their subsequent reduction to amines,[113,286,289,423,435] the use of 5-arylazopyrimidines which are readily reduced to 5-aminopyrimidines,[436-438] and the use of hydrolyzable

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[431] H. Getler, P. M. Roll, J. F. Tinker, and G. B. Brown, *J. Biol. Chem.* **178,** 259 (1949).

[432] M. L. Eidinoff and J. E. Knoll, *J. Am. Chem. Soc.* **75,** 1992 (1953).

[433] J. R. Spies and T. H. Harris, Jr., *J. Am. Chem. Soc.* **61,** 351 (1939).

[434] M. F. Mallette, E. C. Taylor, and C. K. Cain, *J. Am. Chem. Soc.* **69,** 1814 (1947); see also **68,** 1996 (1946).

[435] R. K. Robins, K. J. Dille, C. H. Willits, and B. E. Christensen, *J. Am. Chem. Soc.* **75,** 263 (1953); see correction, p. 6359.

[436] J. Baddiley, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1943,** 386.

[437] B. Lythgoe, A. R. Todd, and A. Topham, *J. Chem. Soc.* **1944,** 315.

[438] L. F. Cavalieri, J. F. Tinker, and A. Bendich, *J. Am. Chem. Soc.* **71,** 533 (1949); correction: **72,** 5801 (1950).

5-acylaminopyrimidines (cf. Cavalieri *et al.*[439]). A new variation employs ethyl nitrosocyanoacetate (or ethyl hydroxyiminocyanoacetate) which, upon condensation with the proper amidine or urea derivative, yields 5-nitrosopyrimidines directly.[440]

Although the 4,5-diaminopyrimidines also serve routinely as intermediates in the synthesis of pteridines,[304,441] their use in purine synthesis presents little ambiguity because of the reagents employed and the much greater reactivity of the 5-amino group. Reaction of 4,5-diamino-2,6-dihydroxypyrimidine with ethyl chloroformate leads only to the 5-urethan, and the urethan (sodium salt) cyclizes to uric acid upon heating.[36] With formic acid, it appears that only the 5-formyl derivatives are formed from di-, tri-, and tetra-aminopyrimidines.[30,36,270,422] When 2,4,5-triamino-6-hydroxypyrimidine is refluxed with formic acid-sodium formate, guanine is formed directly in excellent yield without the necessity of isolating the intermediate 5-formyl compound.[36] Similarly, a nearly quantitative yield of hypoxanthine is obtained merely by refluxing 4,5-diamino-6-hydroxypyrimidine (sulfate) with formic acid.[140] However, other 4-amino-5-formylaminopyrimidines show a reluctance to dehydrate to the corresponding purines, and it is necessary to heat the dry compounds or their sodium or potassium salts at elevated temperatures to effect ring closure. Thus, xanthine, 2,6-diaminopurine, 2-mercaptoadenine, 2-mercaptohypoxanthine, 6-methylpurine, purine, and *N*-alkylxanthines are formed when the dehydrations of the isolated intermediate 5-formyl derivatives are carried out at 152–300°.[30,36,113,182,270,286,442]

For this reason, more suitable techniques of purine formation from 4,5-diaminopyrimidines were sought. In one method, 4,5,6-triaminopyrimidine is smoothly converted by treatment with sodium dithioformate into its 5-thioformyl derivative, and, by boiling in water, hydrogen sulfide is eliminated to furnish adenine.[436] This procedure, which was employed for the preparation of 1,3-N^{15}-labeled adenine, yields a mixture of products 65% of which is adenine and the remainder mainly 4,6-diamino-5-formylaminopyrimidine.[370] In another method, adenine is formed directly by heating 4,5,6-triaminopyrimidine-2-sulfinic and formic acids to 150–160°.[443] The sulfinic acid is obtained by alkaline peroxide oxidation of the 2-mercaptopyrimidine.

A more general method[182] involves the heating (160°) of the sulfates of a number of 4,5-diaminopyrimidines with an equivalent amount of formic acid in excess formamide in a sealed tube. Excellent, and in some cases quantitative, yields of isoguanine, 2,6-diaminopurine, 2-mercapto- and 2-carboxymethylmercapto-adenine, and adenine are obtained in this procedure which is useful in isotopic syntheses.[182,421,438] With the proper sulfates, direct formation of adenine, xanthine, guanine, and 2-methylhypoxanthine occur in excellent yield when only formamide is used.[435,444,445] It is of interest that both formic acid and formamide are required to convert 2-hydroxy-4,5,6-triaminopyrimidine sulfate into isoguanine.[182]

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[439] L. F. Cavalieri, V. E. Blair, and G. B. Brown, *J. Am. Chem. Soc.* **70,** 1240 (1948).

[440] P. D. Landauer and H. N. Rydon, *J. Chem. Soc.* **1953,** 3721.

[441] M. Gates, *Chem. Revs.* **41,** 63 (1947).

[442] J. H. Speer and A. L. Raymond, *J. Am. Chem. Soc.* **75,** 114 (1953).

[443] M. Hoffer, *Jubilee Vol. Dedicated to Emil Christoph Barell*, **1946,** 428.

By the judicious choice of labeled intermediates such as formic acid, urea, thiourea, formamidine, guanidine, cyanoacetic ester, malononitrile, phenylazomalononitrile, acetamidocyanoacetic ester, etc., isotopes have been introduced in nearly every position of purine molecules when the various methods detailed above have been employed. Since C-8-labeled purines may be made suitably from C^{13}- or C^{14}-formic acid in the last step of the synthesis, considerable attention has been drawn to this type of synthesis. It is found that the labeled formyl group of 4,6-diamino-5-formamidopyrimidine sulfate exchanges with nonisotopic formyl groups when the ring closure is carried out in formamide, and a 75% diluted C-8-labeled adenine results.[444] The report[446] of only a 20% dilution when *N*-formylmorpholine was substituted for formamide could not be confirmed[447] since a 50% dilution was found upon reexamination. No dilution of the resulting C-8-labeled adenine occurs when 4,5,6-triaminopyrimidine sulfate is heated with *N*-formyl-C^{14}-morpholine or when the dehydration of 4,6-diamino-5-C^{14}-formamidopyrimidine sulfate is carried out in diethanolamine at 210°.[447] The above dilutions were tentatively explained on the basis of a special type of exchange termed "reversible transformylation" in which formylation of both the 4(or 6)- and 5-amino groups was believed to occur followed by a random deformylation during ring closure. Another explanation lies in the possibility of a lability of carbon 8 in exchange reactions at elevated temperatures (*ca.* 200°) with carbon donors such as formamide. An example of such an exchange is seen in the conversion of uric acid into xanthine by means of hot formamide.[445]

Some of the principles that have been discussed are illustrated below in the synthesis of adenine labeled at N-1 and N-3 with N^{15}, and with C^{13} or C^{14} at C-4, C-6, and C-8:[438,444]

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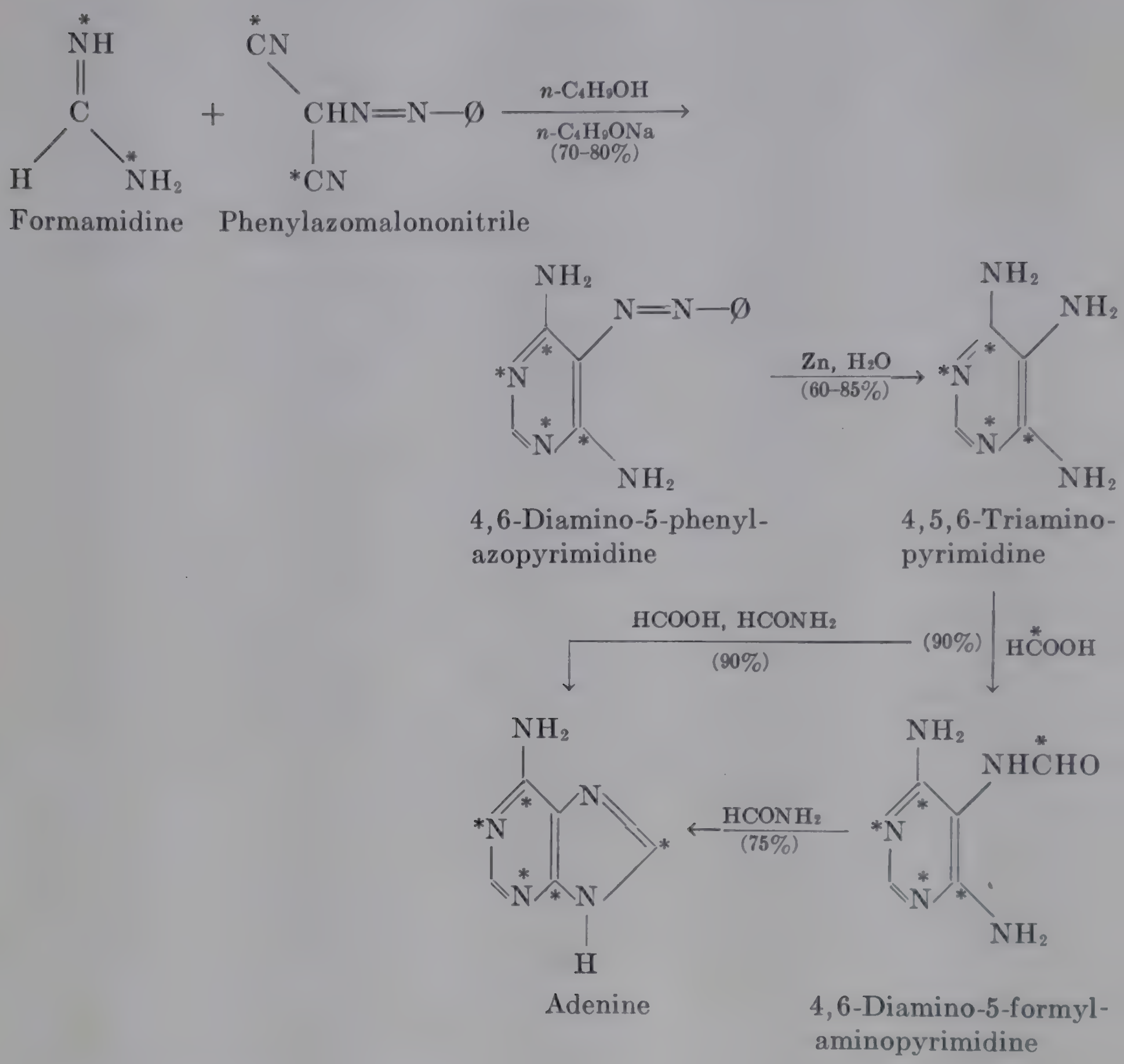
[444] L. F. Cavalieri and G. B. Brown, *J. Am. Chem. Soc.* **71,** 2246 (1949).

[445] H. Bredereck, H.-G. von Schuh, and A. Martini, *Chem. Ber.* **83,** 201 (1950).

[446] V. M. Clark and H. M. Kalckar, *J. Chem. Soc.* **1950,** 1029.

[447] R. Abrams and L. Clark, *J. Am. Chem. Soc.* **73,** 4609 (1951).



c. From Imidazoles

It is of interest that the rat transforms 4-amino-5-imidazolecarboxamide into nucleic acid purines[448] (cf. Chapters 23 and 25) since the conversion of this and other imidazoles into a variety of purines may be achieved chemically as well. In earlier syntheses of purines by this route (cf. Lythgoe[328]), the intermediate imidazoles were relatively inaccessible. This has been remedied to a great extent by newer methods of preparation.[301, 448-452]

The fusion of 4-amino-5-imidazolecarboxamide with urea leads to the formation of xanthine in 75% yield[452] (cf. Stetten and Fox[453]). When the hydrochloride of this imidazole is heated with formamide at 185°, a good yield of hypoxanthine is obtained; formylation yields 4-formamido-5-imidazolecarboxamide, which in turn cyclizes to hypoxanthine by boiling in dilute bicarbonate solution.[452]

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[448] C. S. Miller, S. Gurin, and D. W. Wilson, *Science* **112,** 654 (1950); *J. Am. Chem. Soc.* **74,** 2892 (1952).

[449] I. Heilbron, *J. Chem. Soc.* **1949,** 2099.

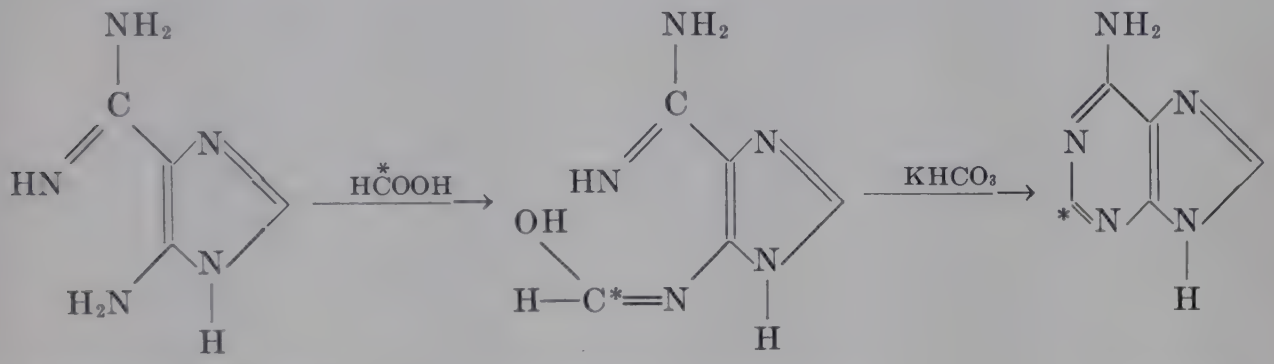
[450] A. H. Cook and I. Heilbron, *Rec. trav. chim.* **69,** 351 (1950).

[451] E. Shaw and D. W. Woolley, *J. Biol. Chem.* **181,** 89 (1949).

[452] E. Shaw, *J. Biol. Chem.* **185,** 439 (1950).

[453] M. R. Stetten and C. L. Fox, Jr., *J. Biol. Chem.* **161,** 333 (1945).

The dihydrochloride of 4-amino-5-imidazolecarboxamidine, prepared by the strong acid hydrolysis of adenine[301] or from malononitrile,[451] gives isoguanine upon fusion with urea or reaction with phosgene.[301,452] Adenine is obtained in 80% yield when the above carboxamidine is formylated and the formyl derivative is cyclized in dilute bicarbonate solution.[452] This reaction has been adapted for the preparation (in 61% yield) of 2-C^{14}-adenine:[454]



4-Amino-5-imidazole-carboxamidine

4-Formamido-5-imidazole-carboxamide (enol form)

Adenine

Addendum

A new antitubercular antibiotic, amicetin ($C_{29}H_{44}N_6O_9$), obtained from *Streptomyces*, has been found to contain, in part, cytosine, *p*-aminobenzoic acid, and *dextro*-D-methylserine.[455]

An inhibitor of *S. faecalis*, 8-aza-6-mercaptopurine has been prepared by the direct thiation of 8-azahypoxanthine with P_2S_5 in boiling pyridine.[456]

Further studies on the anti-tumor activity of 6-mercaptopurine have been carried out.[457] Of 45 children with acute leukemia, treatment with this drug has produced[458] good remission in 15 and partial remission in 10.

The inhibitor 2-thiouracil[253] is incorporated into the ribonucleic acid of tobacco mosaic virus in amounts equal to about 20% of the normal uracil content.[459]

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[454] A. R. P. Paterson and S. H. Zbarsky, *J. Am. Chem. Soc.* **75,** 5753 (1953).

[455] E. H. Flynn, J. W. Hinman, E. L. Caron, and D. O. Woolf, Jr., *J. Am. Chem. Soc.* **75,** 5867 (1953).

[456] C. T. Bahner, B. Stump, and M. E. Brown, *J. Am. Chem. Soc.* **75,** 6301 (1953).

[457] D. A. Clarke, F. S. Philips, S. S. Sternberg, C. C. Stock, G. B. Elion, and G. H. Hitchings, *Cancer Research* **13,** 593 (1953).

[458] J. H. Burchenal, M. L. Murphy, R. R. Ellison, M. P. Sykes, T. C. Tan, L. A. Leone, D. A. Karnofsky, L. F. Craver, H. W. Dargeon, and C. P. Rhoads, *Blood* **8,** 965 (1953).

Exposure of neutral aqueous solutions of uric acid to ultraviolet irradiation gives rise to triuret (1,3-dicarbamylurea).[460]

A detailed paper dealing with the occurrence of 5-hydroxymethylcytosine in the DNA of bacteriophages T_2, T_4, and T_6 of *E. coli* has appeared.[461] The free synthetic base survives treatment with 72% $HClO_4$ for one hour at 100°, but such conditions lead to its destruction when in nucleic acid linkage.

A thoughtful review of the chemistry of the simple pyrimidines has recently been written.[462]

The presence of 2-methyladenine in the vitamin B_{12}-like factor "A" and in crystalline pseudovitamin B_{12d} has been announced.[463,464]

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[462] D. J. Brown, The Simple Pyrimidines, *Rev. Pure Appl. Chem.* **3,** 115 (1953).

[463] F. B. Brown and E. L. Smith, *Biochem. J.*, in press.

[464] H. W. Dion, D. G. Calkins, and J. J. Pfiffner, *J. Am. Chem. Soc.* **76,** 948 (1954).

CHAPTER 4

Chemistry of Nucleosides and Nucleotides*

J. BADDILEY

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* The author has preferred not to follow, in the graphical presentation of structures, the style used by *Chemical Abstracts*—[THE EDITORS].

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I. Nucleosides

1. INTRODUCTION

In most living cells pyrimidines and purines are found in fairly large amounts. [Cf. *Bendich*, Chapter 3.] Occasionally they occur in the free state, e.g., theophylline, theobromine, caffeine, etc.; but those which predominate in normal cells are usually present as glycosides. Purine and pyrimidine glycosides are known as *nucleosides.* The nucleosides may be present as such in cells but are found for the most part as their phosphoric esters, the *nucleotides*, which play a vital role in all cells as coenzymes and, in a highly polymerized condition, as nucleic acids. The term "nucleoside" has been used in two senses. It has been considered as applying only to those pyrimidine and purine glycosides which are formed by hydrolysis of nucleic acids, but it is often used more generally for all naturally occurring pyrimidine and purine glycosides. Nucleosides are, with very few exceptions, either β-D-ribofuranosides or D-2-deoxyribofuranosides. They are generally formed by hydrolysis of nucleic acids with alkaline reagents and may be separated from each other by methods described in Chapters 5 to 8.

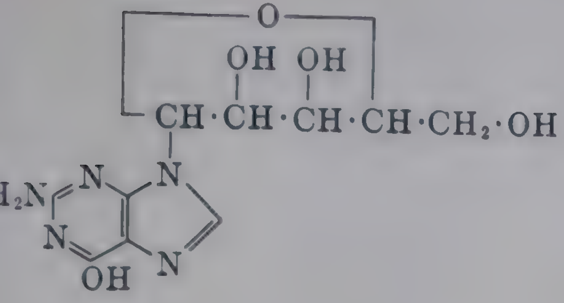
The first nucleoside to be isolated was called vernine[1] and renamed guanosine (I) later. It is found together with adenosine[2] (II) in alkaline hydrolysates of ribonucleic acid. These two substances are believed to represent the entire purine nucleoside components of ribonucleic acids from different sources.

The pyrimidine nucleosides occurring in ribonucleic acid are uridine (III) and cytidine (IV). They are the 3-β-D-ribofuranosides of uracil and cytosine, respectively.

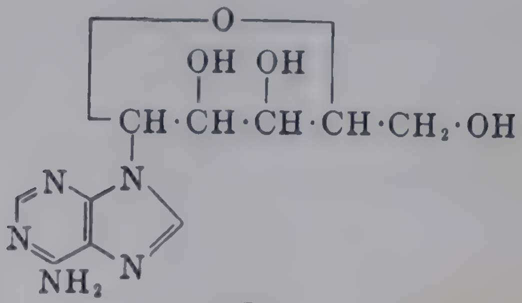
---

[1] E. Schulze and E. Bosshard, *Z. physiol. Chem.* **9**, 420 (1885); **10**, 80 (1885).

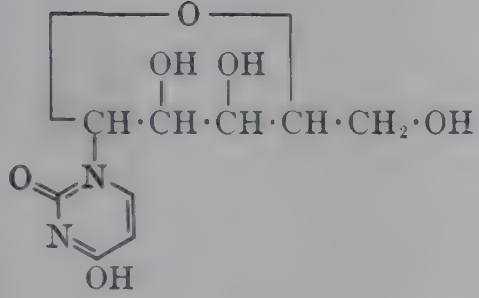
[2] P. A. Levene and W. A. Jacobs, *Ber.* **42**, 2703 (1909).



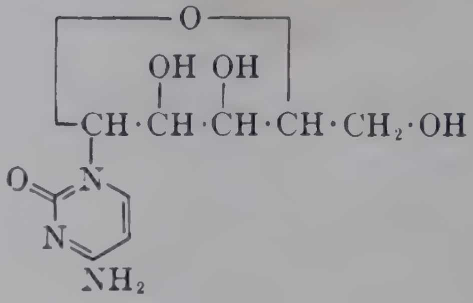
I
Guanosine



II
Adenosine

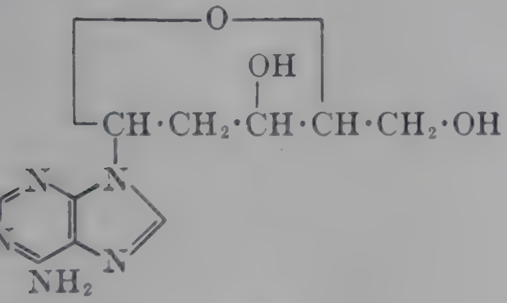


III
Uridine

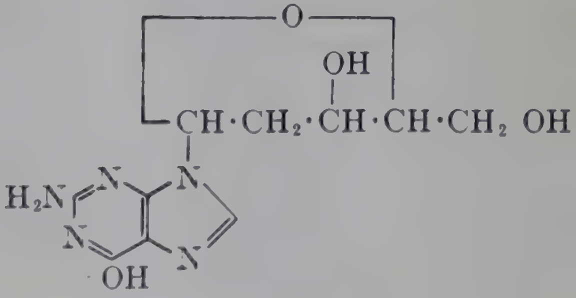


IV
Cytidine

The two purine nucleosides present in deoxyribonucleic acid are adenine
eoxyriboside (V) and guanine deoxyriboside (VI). These are both 9-D-
′-deoxy)ribofuranosides in which the configuration of the glycoside link-
ge is not known.



V
Adenine deoxyriboside



VI
Guanine deoxyriboside

The major pyrimidine nucleoside components of deoxyribonucleic acid
e cytosine deoxyriboside (VII) and thymidine (thymine deoxyriboside)
III). These are both 3-D-(2′-deoxy)ribofuranosides of undetermined con-
guration at the glycosidic linkage. Some samples of deoxyribonucleic acid
ntain small amounts of the pyrimidine nucleosides, 5-methylcytosine
eoxyriboside (IX) and uracil deoxyriboside (X), but it is possible that the
tter is an artifact arising by bacterial deamination of the cytosine nucleo-
de.[3]

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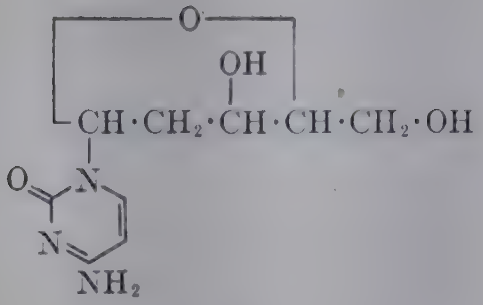
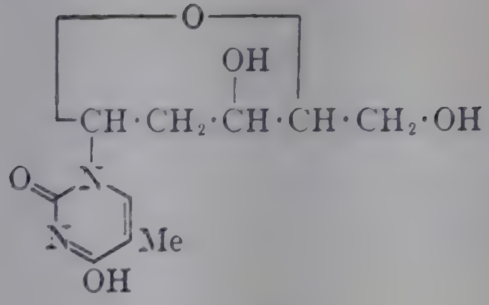
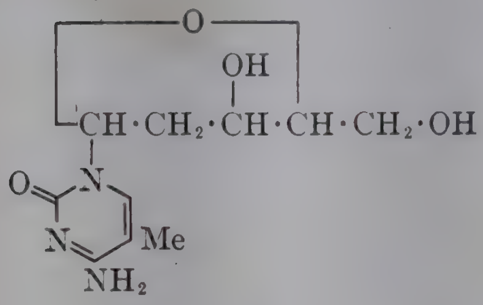
[3] C. A. Dekker and A. R. Todd, *Nature* **166,** 557 (1950).



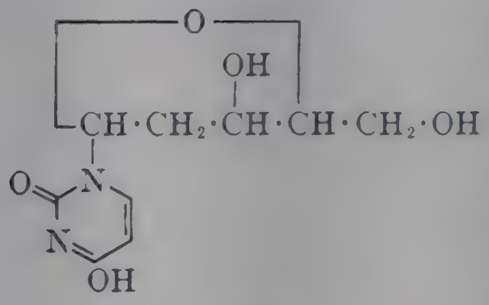
VII
Cytosine deoxyriboside



VIII
Thymidine



IX
5-Methylcytosine deoxyriboside



X
Uracil deoxyriboside

2. STRUCTURE OF NUCLEOSIDES

a. The Nature of the Bases

Adenosine was discovered by Levene and Jacobs[2] as a component of ribonucleic acid. They showed that it yielded adenine (6-aminopurine) on acid hydrolysis. The structure and synthesis of adenine and other purine and pyrimidine bases from nucleic acids are discussed in Chapter 3. Guanine (2-amino-6-hydroxypurine) was identified as the purine component of guanosine by Schulze.[1] The pyrimidine nucleosides uridine and cytidine yield the pyrimidines uracil (2,4-dihydroxypyrimidine) and cytosine (4-amino-2-hydroxypyrimidine), respectively, on vigorous acid hydrolysis.

The deoxyribosides of adenine, guanine, cytosine, and thymine yield the respective purines and pyrimidines after acid hydrolysis.[4, 5]

b. Carbohydrate Components

The carbohydrate component of ribonucleic acid, and hence of the corresponding nucleosides, was described by Hammarsten[6] as a pentose, and its identification as D-ribose followed later. Levene and Jacobs[7-9] obtained it crystalline and reported that it was a new sugar resembling arabinose in many of its properties but differing from both D- and L-arabinose in melting

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[4] P. A. Levene and E. S. London, *J. Biol. Chem.* **81,** 711 (1929); **83,** 793 (1929).

[5] W. Klein, *Z. physiol. Chem.* **224,** 244 (1934); **255,** 82 (1938).

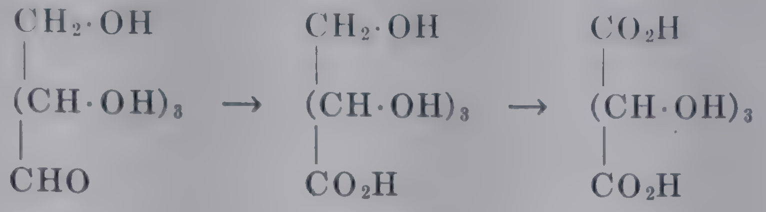
[6] O. Hammarsten, *Z. physiol. Chem.* **19,** 19 (1894).

[7] P. A. Levene and W. A. Jacobs, *Ber.* **41,** 2703 (1908).

[8] P. A. Levene and W. A. Jacobs, *Ber.* **42,** 1198 (1909).

[9] P. A. Levene and W. A. Jacobs, *Ber.* **44,** 746 (1911).

point, rotation, etc. On oxidation it gave, first, D-ribonic acid and then an optically inactive trihydroxyglutaric acid.

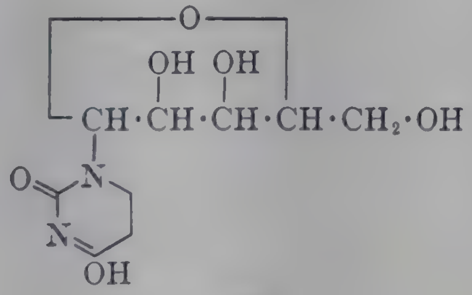


$$\begin{array}{l} CH_2\cdot OH \\ | \\ (CH\cdot OH)_3 \\ | \\ CHO \end{array} \rightarrow \begin{array}{l} CH_2\cdot OH \\ | \\ (CH\cdot OH)_3 \\ | \\ CO_2H \end{array} \rightarrow \begin{array}{l} CO_2H \\ | \\ (CH\cdot OH)_3 \\ | \\ CO_2H \end{array}$$

D-Ribonic acid

Full identification was obtained by the synthesis of D-ribose.[10]

More difficulty was experienced in the identification of the carbohydrate component of the pyrimidine nucleosides. These substances are stable to dilute acids and concentrated acids convert the liberated pentose to furfural. However, simultaneous hydrolysis and oxidation of cytidine with hydrobromic acid and bromine gave 5-bromouracil and D-ribonic acid,[11] indicating that this nucleoside contains a D-ribosyl residue. Furthermore, pyrimidine glycosides may be reduced catalytically to 4,5-dihydropyrimidine glycosides which are quite labile towards acids, giving the pyrimidines and free sugar. Thus uridine is converted into dihydrouridine (XI) and this, after acid hydrolysis, gives D-ribose.[11]



XI
4,5-dihydrouridine

The presence of D-ribose in the four nucleosides obtained from ribonucleic acid has been confirmed by the conversion of their sugar components into D-ribobenziminazole. The suggestion that this type of nucleic acid may contain small amounts of L-lyxose derivatives is now believed to be incorrect.[12]

The sugar component of the deoxyribonucleosides proved very difficult to identify. Acid hydrolysis under conditions similar to those employed with the purine ribonucleosides effects complete conversion of the deoxysugar to levulinic acid. However, short hydrolysis of the guanine deoxynucleoside with very dilute acid gave the base and a crystalline deoxypentose.[4, 13]

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[10] W. A. van Ekenstein and J. J. Blanksma, *Chem. Weekblad* **10,** 664 (1913).

[11] P. A. Levene and F. B. La Forge, *Ber.* **45,** 608 (1912).

[12] J. M. Gulland and G. R. Barker, *J. Chem. Soc.* **1943,** 625; J. M. Gulland, *ibid.* **1944,** 208; G. R. Barker, K. R. Cooke, and J. M. Gulland, *ibid.* **1944,** 339; G. R. Barker, K. R. Farrar, and J. M. Gulland, *ibid.* **1947,** 21.

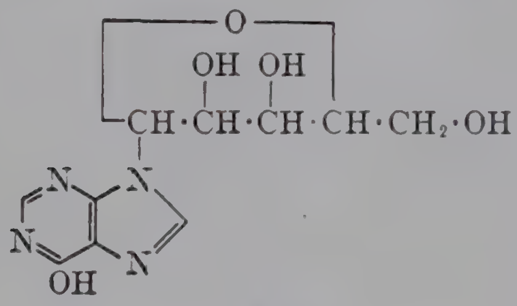
[13] P. A. Levene and T. Mori, *J. Biol. Chem.* **83,** 803 (1929).

It formed a benzylphenylhydrazone, but not an osazone, and gave colo tests typical of the group of 2-deoxysugars. It differed from D-2-deoxyxylos but was indistinguishable from synthetic L-2-deoxyribose in all respect except the sign of rotation.[14] It was then, D-2-deoxyribose. Recently it ha been shown that cautious acid hydrolysis of deoxyribonucleic acid in th presence of α-toluenethiol gives the benzyl mercaptal of D-2-deoxyribos which may be isolated in crystalline form.[15]

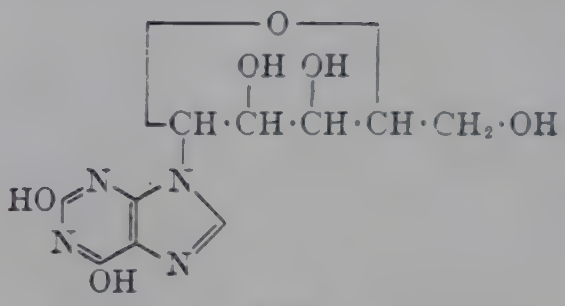
c. *Nucleoside Conversions*

Some degree of generalization about structural features in nucleosides i possible since, although all these features have not always been verified i each nucleoside, the interconversions discussed below enable structural de ductions for one to be applied to others.

Adenosine (II) is converted into inosine (9-D-ribofuranosylhypoxanthine (XII) by the action of nitrous acid.[16-18] Inosine was isolated from natura sources by Haiser and Wenzel in 1908, before its relationship to adenosin and the nucleic acids was established.[19] Similarly, nitrous acid convert guanosine (I) into xanthosine (9-D-ribofuranosylxanthine) (XIII).[16, 20, 2



XII
Inosine



XIII
Xanthosine

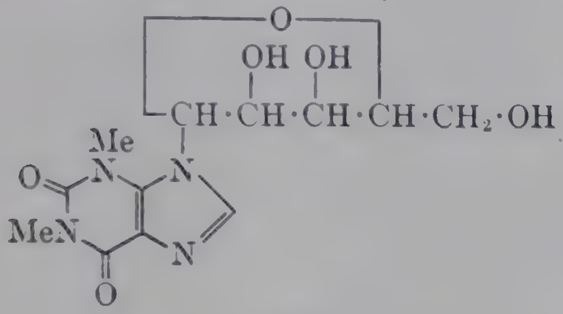
Adenine deoxyriboside is deaminated to hypoxanthine deoxyriboside b an intestinal deaminase.[5] This deaminase is usually present in the enzym concentrate used for the production of pyrimidine and purine deoxyribo sides from deoxyribonucleic acid; consequently early methods for the isola tion of these nucleosides always yielded the hypoxanthine and not th adenine derivative.[4] The action of the deaminase may be inhibited by silve ions.

[14] P. A. Levene, L. A. Mikeska, and T. Mori, *J. Biol. Chem.* **85,** 785 (1930).
[15] P. W. Kent, *Nature* **166,** 442 (1950).
[16] P. A. Levene and W. A. Jacobs, *Ber.* **43,** 3150 (1910).
[17] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **111,** 313 (1935).
[18] J. M. Gulland and E. R. Holiday, *J. Chem. Soc.* **1936,** 765.
[19] F. Haiser and F. Wenzel, *Monatsh.* **29,** 157 (1908).
[20] J. M. Gulland and T. F. Macrae, *J. Chem. Soc.* **1933,** 662.
[21] P. A. Levene, *J. Biol. Chem.* **55,** 437 (1923).

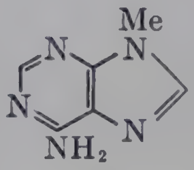
The pyrimidine ribonucleosides have been related by the conversion of cytidine into uridine with nitrous acid.[16]

d. Position of the Glycosidic Linkage

The ease with which purine nucleosides may be hydrolyzed in acid strongly suggests that the linkage between purine and sugar does not involve a C—C bond, but is much more in keeping with the known properties of *N*-glycosides. In view of the nucleoside conversions outlined above, the primary amino groups in adenosine and guanosine cannot be involved. It follows, then, that the N atoms at positions 1, 3, 7, or 9 may be involved. Positions 1 and 3 were eliminated by methylation of xanthosine to give a theophylline riboside (XIV).[21]



XIV
Theophylline riboside



XV
9-Methyladenine

The early work established that the sugar was attached to one of the iminazole N atoms in the purine nucleosides. The assumption that these were 7-glycosides was corrected later. The ultraviolet absorption spectra of adenosine and inosine closely resemble those of 9-methyladenine (XV) and 9-methylhypoxanthine but differ markedly from those of the corresponding 7-methyl derivatives.[18] Similarly, guanosine[22] and xanthosine[23] show ultraviolet absorption spectra very similar to those of the corresponding 9-methyl and not the 7-methyl derivatives. It was concluded that the natural purine ribosides are 9- and not 7-glycosides.

This technique has also been applied to the purine deoxyribosides. Adenine deoxyriboside[24] and guanine deoxyriboside[22] show spectra very similar to those of adenosine and guanosine, and it was concluded that these must be 9-glycosides.

Chemical evidence supporting the 9-glycosidic structure of the purine ribosides has been forthcoming from the periodate oxidation studies discussed in the next section.

Chemical methods have been used for determining the position of the glycosidic linkage in the pyrimidine ribosides. Since cytidine may be deaminated to uridine without loss of the ribose residue it follows that the glycosidic linkage does not involve the 6-substituent in these nucleosides. Furthermore, position 5 may be dismissed from consideration since uridine can be converted into 5-nitrouridine and 5-bromouridine without loss of the carbohydrate residue. The formation of 4,5-diphenylhydrazinouridine (XVI) by the action of bromine, then phenylhydrazine, on uridine indicates the absence of substituents on positions 4 and 5.[25] This reaction is considered specific for 3-substituted uracil derivatives; consequently it seemed likely that uridine and cytidine are 3-glycosides.

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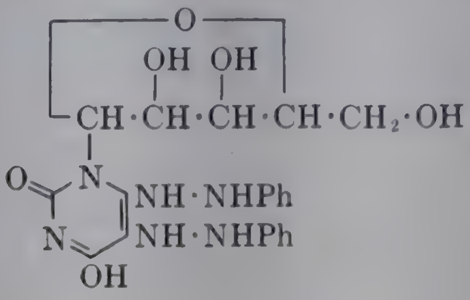
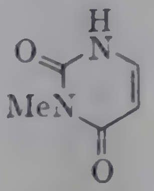
[22] J. M. Gulland and L. F. Story, *J. Chem. Soc.* **1938,** 692.

[23] J. M. Gulland, E. R. Holiday, and T. F. Macrae, *J. Chem. Soc.* **1934,** 1639.

[24] J. M. Gulland and L. F. Story, *J. Chem. Soc.* **1938,** 259.



XVI
Diphenylhydrazinouridine



XVII
1-Methyluracil

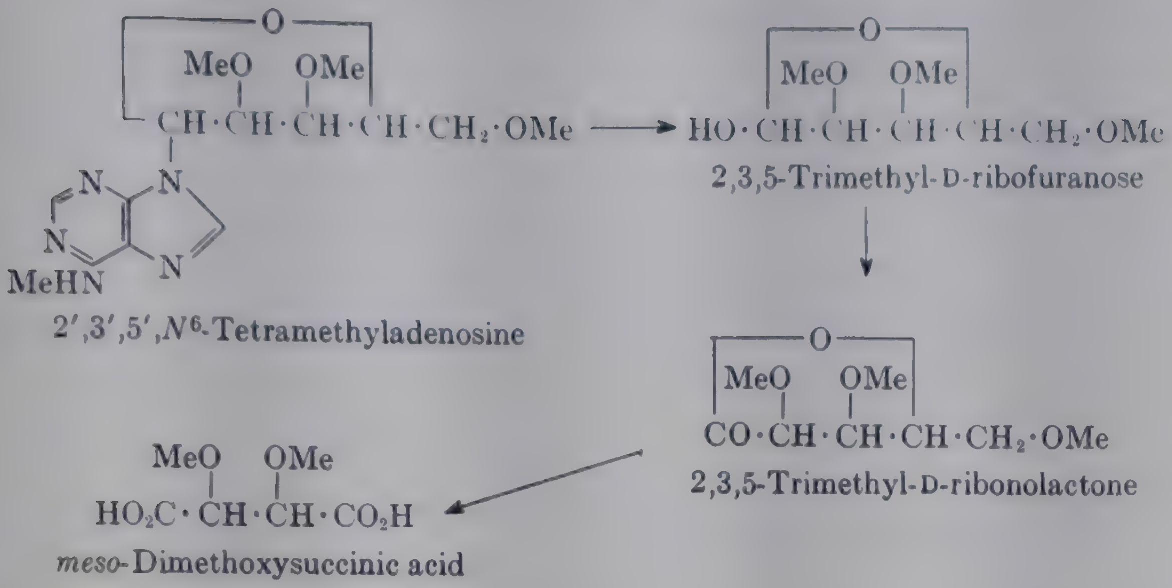
The reaction between uridine and hydrazine to give pyrazolone also indicates the absence of substituents at position 4.[26] Since uridine may be converted into N-methyluridine which, on hydrolysis, yields 1-methyluracil (XVII), it follows that uridine and hence cytidine are 3-glycosides.[27]

Thymidine is probably a 3-glycoside since methylation and hydrolysis of deoxyribonucleic acid results in the production of 1-methylthymine.[28]

e. The Ring Structure of the Carbohydrates

The size of the sugar ring in the purine nucleosides has been determined by methylation and oxidation. Acetylation of adenosine and subsequent methylation then deacetylation yields a trimethyl-N-methyladenosine and this, on hydrolysis with dilute acid, gives N^6-methyladenine and a trimethylribose.[29] The same trimethylribose may be isolated after similar transformations on guanosine.[30] Its structure is established by oxidation to 2,3,5-trimethyl-γ-D-ribonolactone and then to *meso*-dimethoxysuccinic acid. The trimethylribose itself was identified subsequently by comparison with synthetic 2,3,5-trimethyl-D-ribofuranose.[31]

[25] P. A. Levene, *J. Biol. Chem.* **63,** 653 (1925).
[26] P. A. Levene and L. W. Bass, *J. Biol. Chem.* **71,** 167 (1926).
[27] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **104,** 385 (1935).
[28] H. Bredereck, G. Müller, and E. Berger, *Ber.* **73,** 1058 (1940).
[29] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **94,** 809 (1932).
[30] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **97,** 491 (1932).
[31] P. A. Levene and E. T. Stiller, *J. Biol Chem.* **102,** 187 (1933).



2′,3′,5′,N^6-Tetramethyladenosine

2,3,5-Trimethyl-D-ribofuranose

2,3,5-Trimethyl-D-ribonolactone

meso-Dimethoxysuccinic acid

The ring structure of the ribose residue in the pyrimidine nucleosides has been determined in much the same way. Hydrogenation of triacetyluridine gives the 4,5-dihydro derivative which may be methylated with simulataneous deacetylation, then oxidized with bromine and hydrogen bromide. The product is 2,3,5-trimethyl-γ-D-ribonolactone, which may be isolated and identified as before.[32] It follows that uridine and cytidine are furanosides.

The furanose configuration of ribonucleosides is supported by their behavior towards trityl chloride. It is known that this reagent reacts preferentially with primary hydroxyl groups in sugars and their derivatives, giving trityl ethers. Exceptions to the rule are known, but under controlled conditions reaction is indicative of the presence of a primary hydroxyl group in a sugar. Adenosine gives a mixture of mono- and ditrityladenosine under these conditions.[33-36] Monotrityladenosine gives a tritosyl derivative which is converted into tritosyladenosine by short acid hydrolysis. None of the tosyl groups in this compound is replaceable by iodine when heated with sodium iodide. Since this replacement is usually confined to tosyl esters of primary alcohols it follows that tritosyladenosine bears an unsubstituted primary hydroxyl group, i.e., it must be N^6,2′,3′-tritosyladenosine. The monotrityladenosine must be 5′-monotrityladenosine and consequently adenosine must contain a furanose ring. The ditrityladenosine is N^6,5′-ditrityladenosine, as shown by transformations on its diacetyl derivative. Hydrolysis of the last-named compound with acetic acid yields a diacetyladenosine (2′,3′-diacetyladenosine), identical with that obtained by hydrolysis of N^6,2′,3′-triacetyl-5′-trityladenosine.

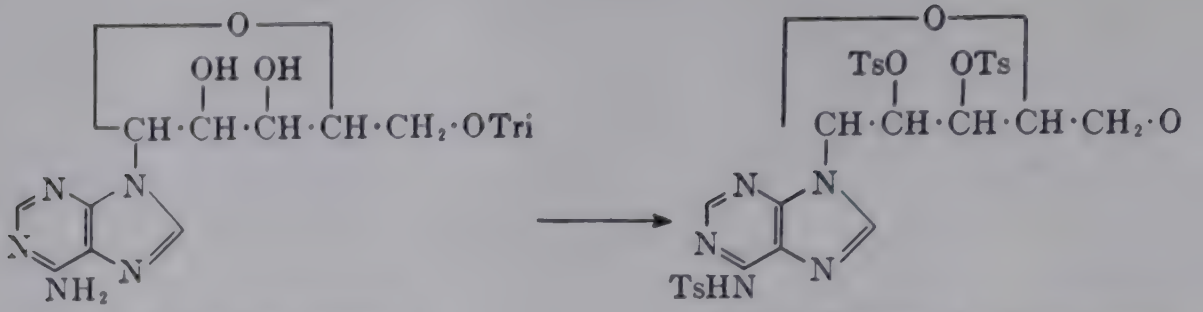
[32] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **101,** 529 (1933).

[33] H. Bredereck, *Ber.* **66,** 198 (1933).

[34] H. Bredereck, *Z. physiol. Chem.* **223,** 61 (1934).

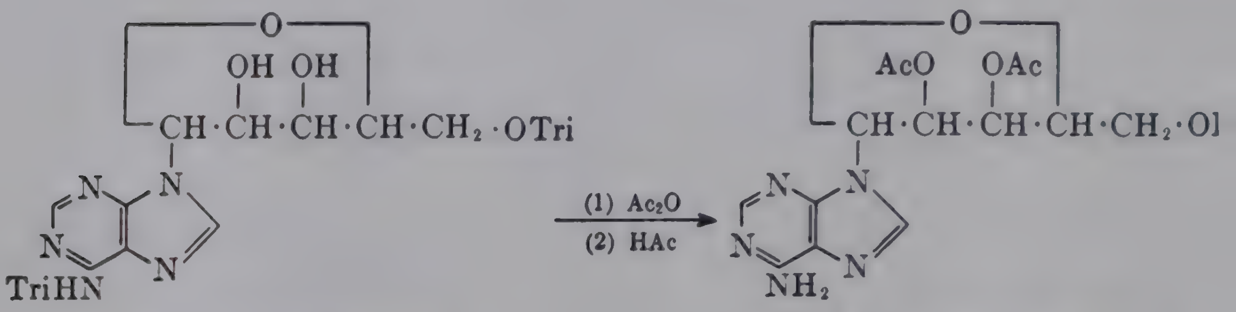
[35] H. Bredereck, *Ber.* **65,** 1830 (1932).

[36] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **121,** 131 (1937).



5′-Trityladenosine

N^6,2′,3′-Tritosyl-5′-trityladenosin



N^6,5′-Ditrityladenosine

2′,3′-Diacetyladenosine

The structure of 2′,3′-diacetyladenosine is confirmed by reaction wit *p*-toluenesulfonyl chloride, giving the N^6,5′-ditosyl derivative. Only on tosyloxy group in this compound may be replaced by iodine on treatmen with sodium iodide.[36]

Similar transformations have established the furanose structure for uridine. Uridine forms mono- and ditrityl derivatives.[27, 35, 37] Methylatio then hydrolysis, of monotrityluridine gives 2′,3′-dimethyluridine. The latter substance forms a monotosyl derivative which reacts readily with sodium iodide to give crystalline 5′-iodo-2′,3′-dimethyluridine. It follow that the original trityl compound is 5′-trityluridine. Also, tosylation of thi compound gives 2′,3′-ditosyl-5′-trityluridine, which after hydrolysis of th trityl residue does not react with sodium iodide.

Tritylcytidine[33, 38] and tritylguanosine[39] are probably 5′-trityl derivatives. In view of the insolubility of guanosine in pyridine the trityl derivative must be prepared by an indirect method.

Thymidine forms a monotrityl derivative.[40] This is believed to be 5′-tr tylthymidine since it may be converted into a monotosyl compound (3 tosyl-5′-tritylthymidine) which does not react with sodium iodide. It fo lows that thymidine possesses a furanose ring.

Further support for the furanose configuration of thymidine is obtaine from the observation that, unlike guanosine and inosine, it does not increas

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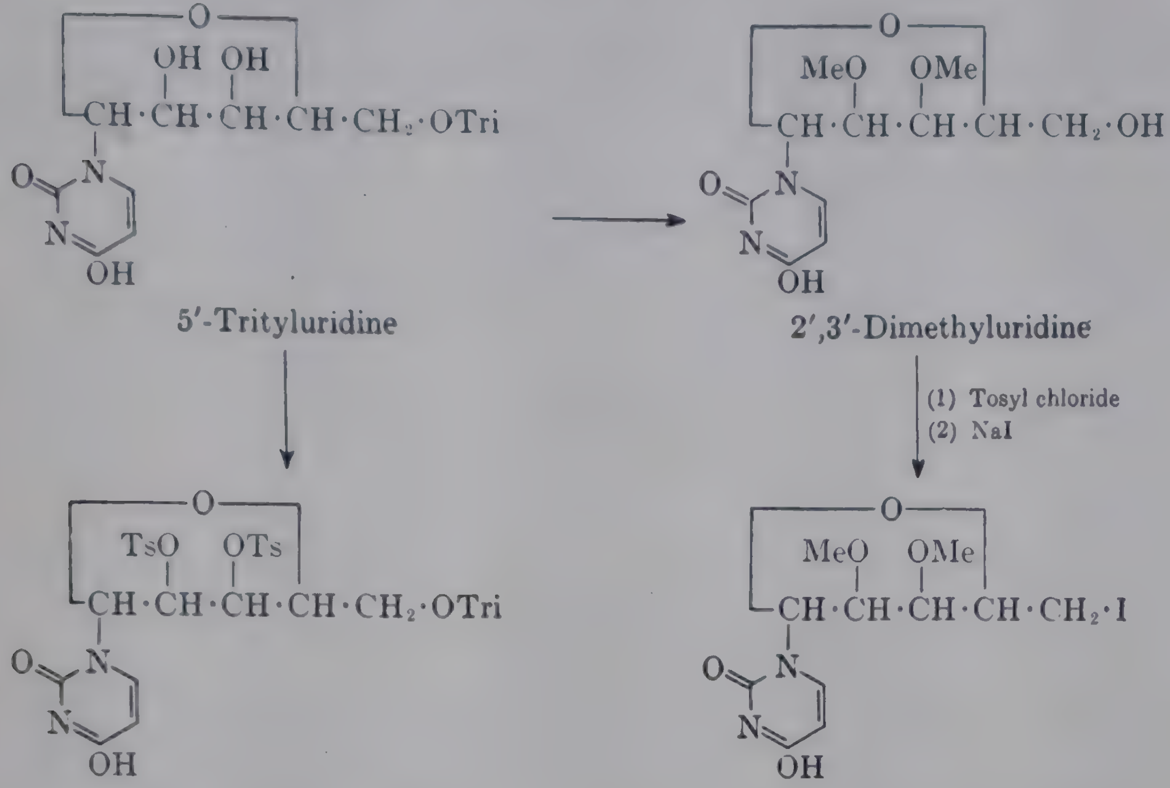
[37] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **105,** 419 (1934).

[38] H. Bredereck, E. Berger, and J. Ehrenberg, *Ber.* **73,** 269 (1940).

[39] H. Bredereck and E. Berger, *Ber.* **73,** 1124 (1940).

[40] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **109,** 623 (1935).



5′-Trityluridine

2′,3′-Dimethyluridine

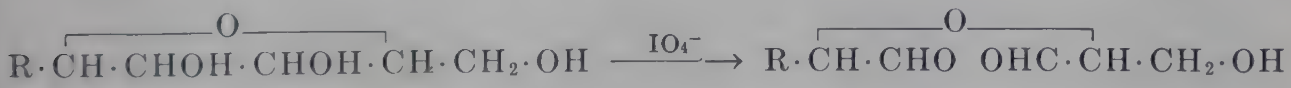
(1) Tosyl chloride
(2) NaI

2′,3′-Ditosyl-5′-trityluridine

5′-Iodo-2′,3′-dimethyluridine

the acidity of boric acid in the Böeseken test[41] and consequently does not possess a *cis*-1,2-glycol grouping. Guanine and hypoxanthine deoxyribosides resemble thymidine in this respect and so are probably furanosides.[42]

Strong evidence for the furanose structure in both ribonucleosides and deoxyribonucleosides is provided by the action of periodate on these compounds. The course of oxidation of nucleosides with periodate follows a course similar to the corresponding reactions of the *O*-glycosides. The natural ribonucleosides consume 1 mol. of periodate, giving a dialdehyde but no formic acid. This behavior is consistent with a furanoside structure.[43]



$$\mathrm{R\cdot CH\cdot CHOH\cdot CHOH\cdot CH\cdot CH_2\cdot OH \xrightarrow{IO_4^-} R\cdot CH\cdot CHO \quad OHC\cdot CH\cdot CH_2\cdot OH}$$

Under similar conditions the pyrimidine and purine deoxyribosides do not consume periodate. The *cis*-1,2-glycol system must be absent from these compounds which must be furanosides.[44]

Adenosine, guanosine,[36] inosine[17] and uridine[45] are converted into 2′,3′-isopropylidene derivatives by reaction with acetone in the presence of acidic dehydrating agents, e.g., zinc chloride, copper sulfate, sulfuric acid. Both 2′,3′-isopropylidene inosine and 2′,3′-isopropylidene uridine form 5′-tosyl derivatives which yield the corresponding 5′-iodo derivatives by reactio with sodium iodide. These conversions, like those already discussed for th trityl derivatives, are consistent with furanose ring structures.

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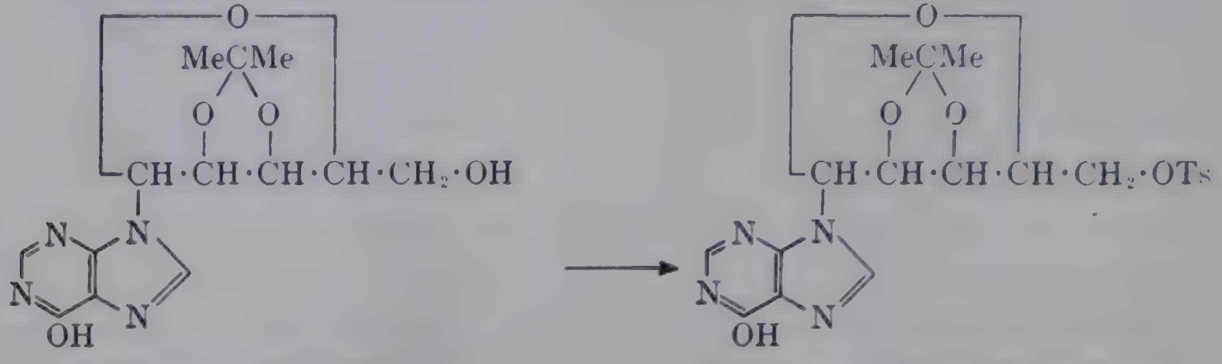
[41] P. A. Levene and R. S. Tipson, *Z. physiol. Chem.* **234,** V (1935).

[42] K. Makino, *Biochem. Z.* **282,** 263 (1935).

[43] B. Lythgoe and A. R. Todd, *J. Chem. Soc.* **1944,** 592.

[44] D. M. Brown and B. Lythgoe, *J. Chem. Soc.* **1950,** 1990.

[45] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **106,** 113 (1934).



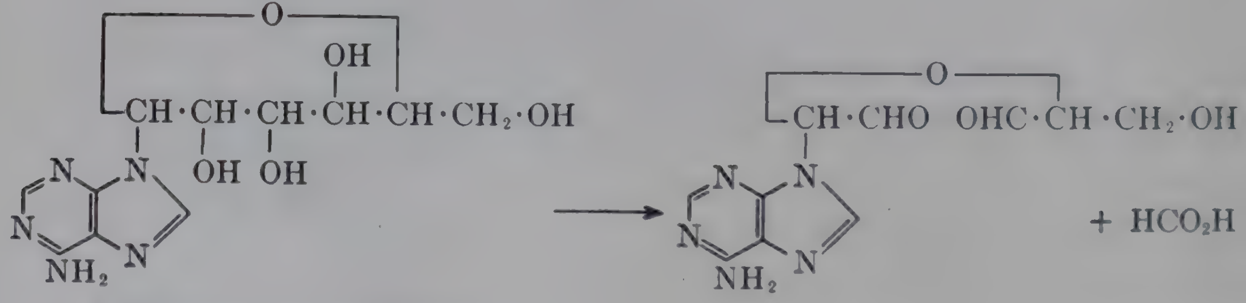
2′,3′-Isopropylidene inosine

2′,3′-Isopropylidene-5′-tosylinosine

f. Configuration of the Glycosidic Linkage

The behavior of ribonucleosides towards periodate has been used in chemical determination of the configuration of their glycosidic linkage and at the same time, provides confirmatory evidence for the location of th sugar at position 9 in the purine nucleosides.

The dialdehyde obtained by the action of periodate on adenosine i identical with the dialdehyde from 9-D-mannopyranosyladenine.[46] Thi mannosyl compound was prepared by the synthetic route outlined on pag 153 and so must be a 9-glycoside. It follows that adenosine is also a 9-gly coside.



9-D-Mannopyranosyladenine

Periodate oxidation of a glucosyladenine also gives this dialdehyde.[4 This glucoside almost certainly has the β-configuration since it was synthe sized from α-acetobromoglucose, and it is reasonable to expect inversio during the formation of glucosides from this compound. The optical proper ties of the oxidation product are consistent with this view. It follows tha the natural nucleoside must also have the β-configuration.

In the same way, the dialdehydes obtained by periodate oxidation c uridine and cytidine are identical with those from synthetic samples c

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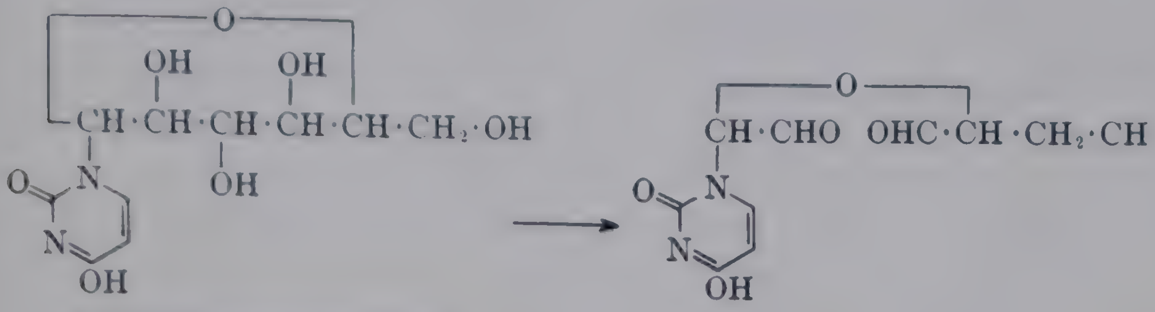
[46] B. Lythgoe, H. Smith, and A. R. Todd, *J. Chem. Soc.* **1947,** 355.

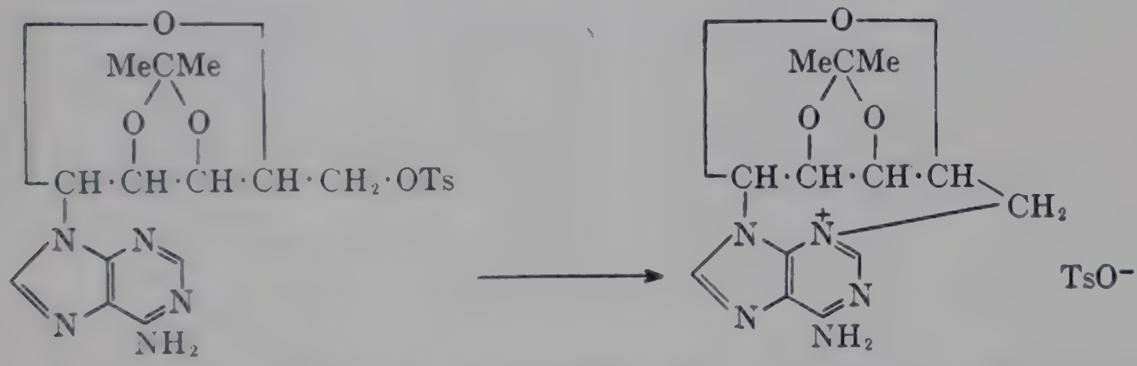
[47] J. Davoll, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1944,** 833.

3-β-D-glucopyranosyluracil and 3-β-D-glucopyranosylcytosine, respectively. The glucosides were prepared by the synthetic route described on page 155 and must be 3-β-derivatives.



3-β-D-Glucopyranosyluracil

The β-configuration of the natural nucleosides is also shown by the behavior of the 5′-tosyl derivatives of the basic members, adenosine and cytidine. Both 2′,3′-isopropylidene-5′-p-toluenesulfonyladenosine and the corresponding cytidine derivative isomerize with great ease to the respective cyclonucleosides.[48] Steric requirements for these transformations are only satisfied by β-glycosides.



X-ray analysis of several nucleosides has confirmed the structures already assigned on chemical evidence. In addition it appears that the glycosidic linkage in adenosine lies in the same plane as the purine ring. The purine ring is flat, in agreement with its aromatic properties, whereas the sugar is slightly nonplanar. The sugar ring lies approximately perpendicular to the purine ring in adenosine.[49] Certain other features have been noted. Although the examination was incomplete, other nucleosides seem to conform to the same general pattern. [Cf. *Jordan*, Chapter 13.]

3. SYNTHESIS OF NUCLEOSIDES

a. Purine Nucleosides

A number of purine glycosides was synthesized by Emil Fischer and his collaborators. These were mainly derivatives of theophylline, theobromine, and 2,6,8-trichloropurine and were prepared from the silver salts of th appropriate purines and acetohalogeno-sugars. The products were deacety ated with ammonia in methanol.[50] Theophylline and theobromine hexo sides,[50, 51] pentosides,[51-55] methyl pentosides,[56-58] a glucodesoside,[59] and rhamnofuranoside[60] may be prepared in this way. A comparison of th absorption spectra of these substances with known derivatives of theophy line and theobromine indicates that the theophylline derivatives are 7-gly cosides and the theobromine derivatives are *O*-glycosides.[23] However, th silver salts of 2,6,8-trichloropurine and 2,8-dichloroadenine also reac with acetohalogenosugars[50] giving acetylated glycosides. The 2,8-dichloro adenine glucoside is a 9-glucopyranoside as is shown by its absorption spec trum[24] and by periodate oxidation. The deacetylated glucoside may b converted into 9-D-glucopyranosyl-adenine or -guanine by appropriat substitution of the chlorine atoms as shown.

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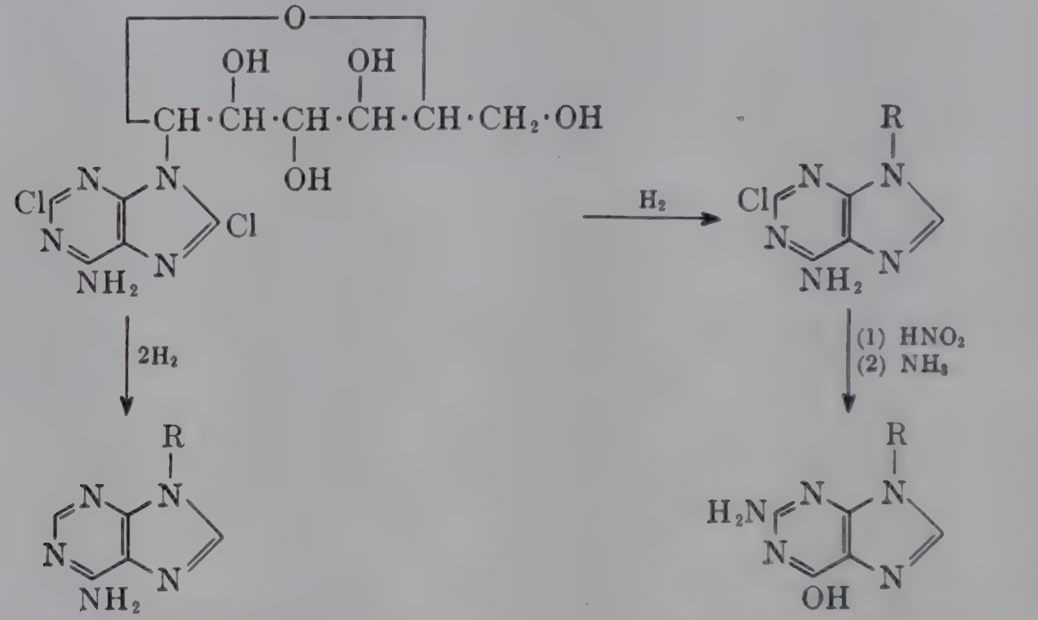
[48] V. M. Clark, A. R. Todd, and J. Zussman, *J. Chem. Soc.* **1951,** 2952.

[49] S. Furberg, *Acta Chem. Scand.* **4,** 751 (1950).



9-D-Glucopyranosyladenine

9-D-Glucopyranosylguanine

In order to apply this method of synthesis to the natural purine nucleo sides an acetohalogenoribofuranose is required. The bromo compound i prepared from 5-trityl-D-ribose, through 5-trityl-1,2,3-triacetylribose, fron which the trityl group may be removed by hydrogenolysis. The resulting 1,2,3-triacetyl-D-ribofuranose is converted on acetylation into 1,2,3,5-tetraacetyl-D-ribofuranose and hence into acetobromoribofuranose.[54] β-Tetraacetyl-D-ribofuranose is also stated to be formed by acetylation of D-ribose at elevated temperatures.[61]

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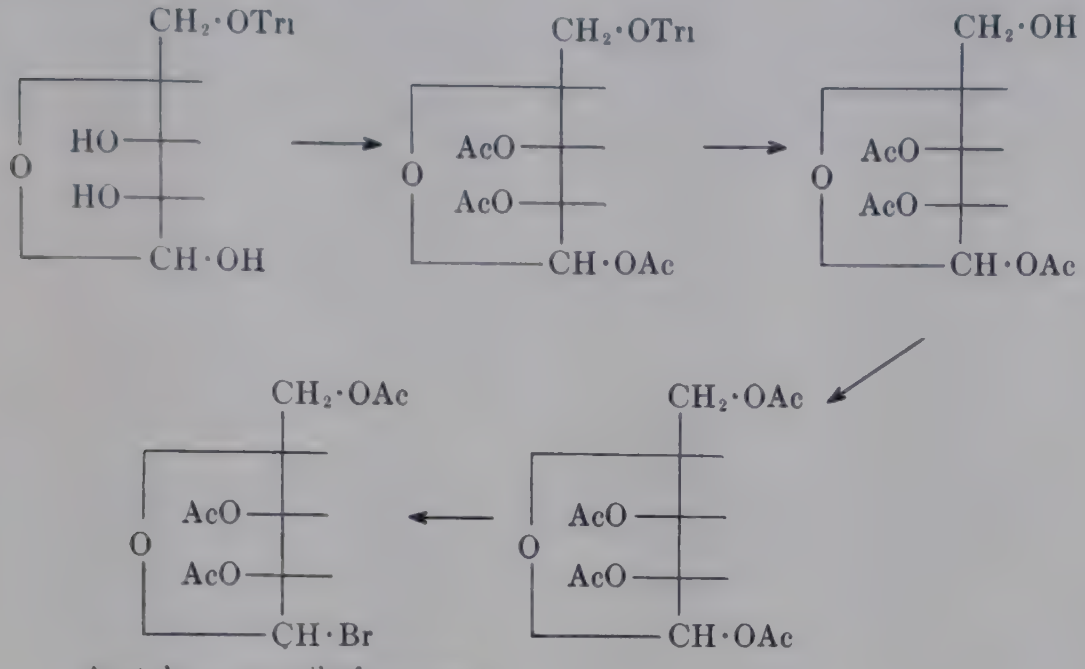
[50] E. Fischer and B. Helferich, *Ber.* **47,** 210 (1914).
[51] B. Helferich and M. von Kühlewein, *Ber.* **53,** 17 (1920).
[52] E. Fischer, *Ber.* **47,** 1377 (1914).
[53] J. Pryde and R. T. Williams, *J. Chem. Soc.* **1933,** 640.
[54] G. A. Howard, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1947,** 1052.
[55] P. A. Levene and H. Sobotka, *J. Biol. Chem.* **65,** 463 (1925).
[56] E. Fischer, B. Helferich, and P. Ostmann, *Ber.* **53,** 873 (1920).
[57] E. Fischer and K. von Fodor, *Ber.* **47,** 1058 (1914).
[58] P. A. Levene and J. Compton, *J. Biol. Chem.* **117,** 37 (1937).
[59] P. A. Levene and F. Cortese, *J. Biol. Chem.* **92,** 53 (1931).
[60] P. A. Levene and J. Compton, *J. Biol. Chem.* **114,** 9 (1936).



Acetobromo-D-ribofuranose

Repetition of the nucleoside syntheses described above with 2,6-dichloroadenine and acetochloro-D-ribofuranose (prepared in an analogous manner) gives adenosine[62] and guanosine.[63] Acetobromo-D-arabofuranose is prepared similarly, but this gives α-glycosides with theophylline and adenine.[64] Xylofuranosides may also be prepared by this route. This synthetic method is not readily applicable to the silver salt of adenine itself since the basicity of the purine is sufficient to effect dehydrohalogenation of the sugar derivative. The difficulty may be overcome by acetylation or benzoylation of the amino groups. From the chloromercury salt of 2,6-diacetamidopurine and acetochlororibofuranose an acetylated 9-D-ribofuranoside is obtained. Acetyl groups may be removed readily from this compound giving 9-D-ribofuranosyl-2,6-diaminopurine. Partial removal of acetyl groups followed by deamination with nitrous acid then complete deacetylation gives guanosine in good yield.[65] Adenosine is prepared in an analogous manner and deamination of the diaminopurine riboside gives crotonoside (see p. 158).[66]

[61] H. Zinner, *Ber.* **83,** 153 (1950).

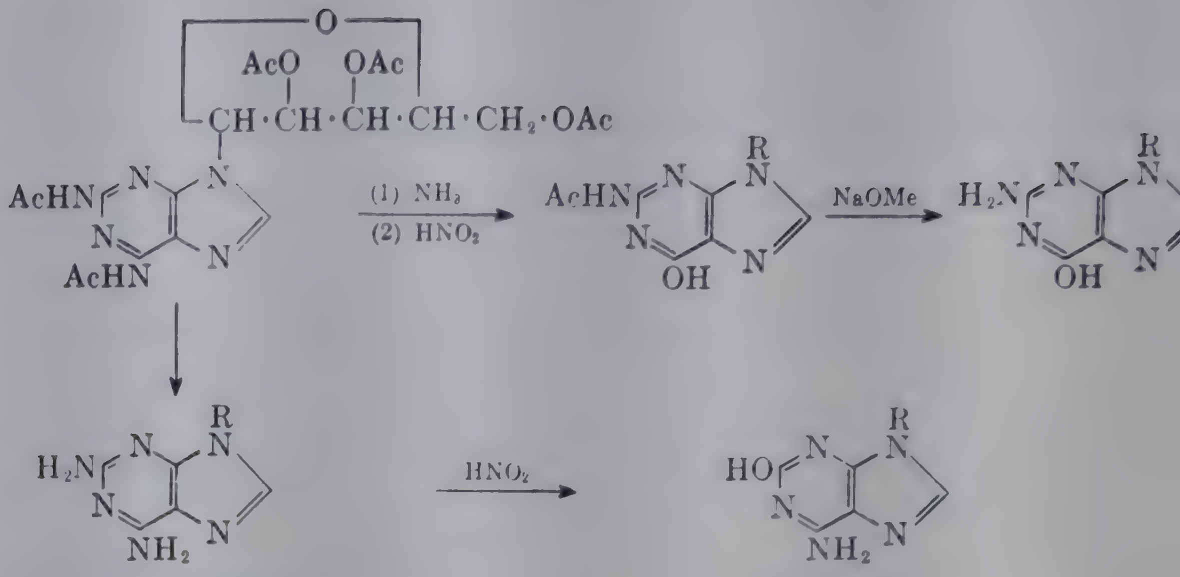
[62] J. Davoll, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1948,** 967.

[63] J. Davoll, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1948,** 1685.

[64] N. W. Bristow and B. Lythgoe, *J. Chem. Soc.* **1949,** 2306; P. Chang and B. Lythgoe, *ibid.* **1950,** 1992.

[65] J. Davoll and B. A. Lowy, *J. Am. Chem. Soc.* **73,** 1650 (1951).

[66] J. Davoll, *J. Am. Chem. Soc.* **73,** 3174 (1951).


9-D-Ribofuranosyl-2,6-diaminopurine

Crotonoside

The above syntheses are moderately convenient and were valuable fo demonstrating the β-configuration of the glycosidic linkage in the natura nucleosides. However, they do not in themselves establish the position c attachment of sugar and purine. An alternative synthesis, although les convenient, is unambiguous and may be regarded as a modification of general synthesis for purines and their 9-substituted derivatives.[67, 68] I the scheme described here the starting point is a substituted 4,6-diaminopyrimidine. For the synthesis of purine glycosides a 4-amino-6-glycosy aminopyrimidine is employed. These may be prepared by acid-catalyze condensation of a diaminopyrimidine with a sugar in alcoholic solution. A 2′,4′- or 2′,5′-dichlorophenylazo group is introduced at the 5-position i the pyrimidine ring by coupling in neutral solution with the appropriat diazonium salt.[70] The hydroxyl groups in the sugar residue are usuall acetylated at this stage and the azo group reduced to amino with hydroge and a nickel catalyst. The resulting acetylated 4,5-diamino-6-glycosy aminopyrimidine is converted into the corresponding 5-thioformamidopyrimidine by reaction with dithioformic acid or its sodium salt. Cyclizatio of the thioformamido compound in pyridine may proceed in two directions giving the acetylated 9-glycosyladenine or 6-glycosylaminopurine, whic can be separated before or after deacetylation.[71]

The yield of purine glycoside obtained in the above synthesis is improve by cyclization of the thioformamido compound with alcoholic alkoxid solutions. Under these conditions unacetylated glycosylpyrimidines giv exclusively the 9-glycosylpurines whereas the acetylated derivatives yiel

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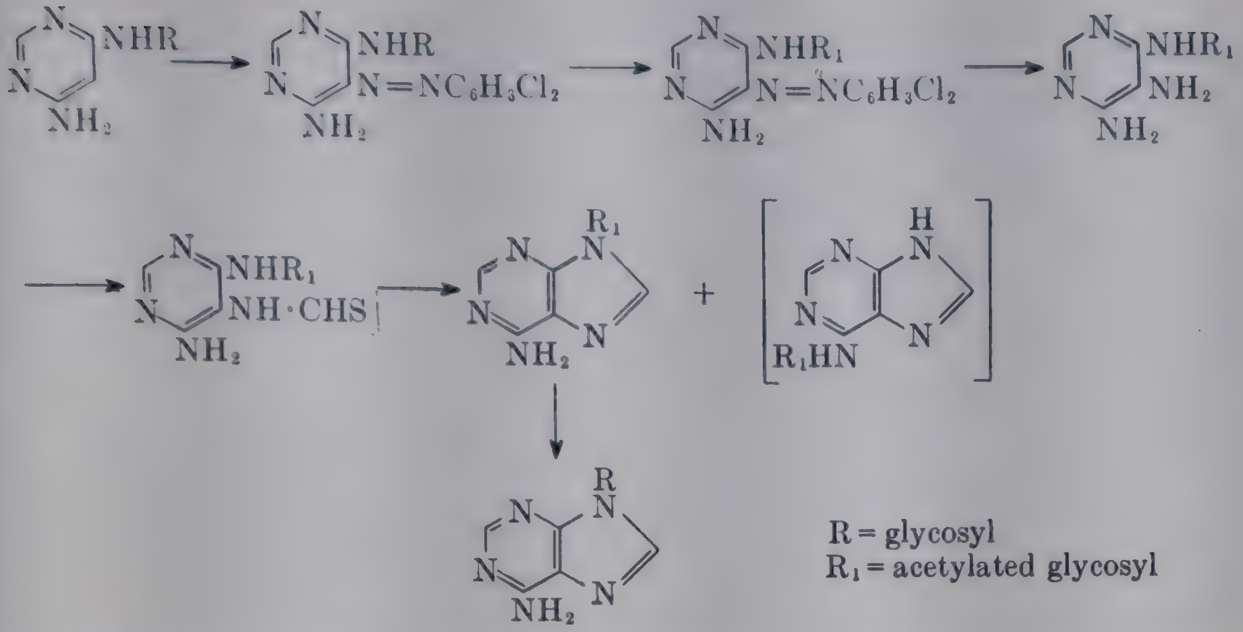
[67] J. Baddiley, B. Lythgoe, D. McNeil, and A. R. Todd, *J. Chem. Soc.*, **1943**, 383.

[68] J. Baddiley, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1943**, 386.

[69] J. Baddiley, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1943**, 571.

[70] B. Lythgoe, A. R. Todd, and A. Topham, *J. Chem. Soc.* **1944**, 315.

[71] J. Baddiley, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1944**, 318.



R = glycosyl
R_1 = acetylated glycosyl

mixtures of 9-glycosides and 7-glycosylaminopurines.[72] The general method has been used for the synthesis of 9-D-xylopyranosyl-,[73] 9-D-ribopyranosyl-,[74] 9-D-mannopyranosyl-,[46] 9-D-glucopyranosyl-adenine,[75] 9-D-xylopyranosyl-2-methylthioadenine,[76] and 9-D-glucopyranosylisoguanine.[77]

Whereas the glycosides prepared by the general route are undoubtedly 9-β-glycosides,[46, 47, 78, 79] periodate titration shows that they are pyranosides. In order to ensure a furanose structure in the glycosidic residue it is necessary to start with a suitably protected aldehydo-sugar. For the synthesis of adenosine[80] along these lines 2,3,4-triacetyl-5-benzyl-D-ribose is condensed with 4,6-diamino-2-methylthiopyrimidine to give a Schiff base. A methylthio residue at position 2 is necessary to increase the reactivity of the aminopyrimidine. After removal of the acetyl groups with methanolic ammonia rearrangement occurs to the glycofuranoside. This is coupled with diazotized 2,5-dichloroaniline, acetylated, and then reduced with zinc and acetic acid to the amine, thioformylated, and cyclized. The 2-methylthio and 5′-benzyl groups are removed by hydrogenolysis with nickel, then acetyl groups removed with sodium methoxide, giving adenosine.

It is important in this synthesis to protect the 5-position in the initial sugar derivative with a benzyl or similar group. When acetyl or benzoyl groups are used in this connection, mixtures of furanosides and pyranosides

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[72] G. W. Kenner and A. R. Todd, *J. Chem. Soc.* **1946,** 852.

[73] G. W. Kenner, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1944,** 652.

[74] J. Baddiley, G. W. Kenner, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1944,** 657.

[75] A. Holland, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1948,** 965.

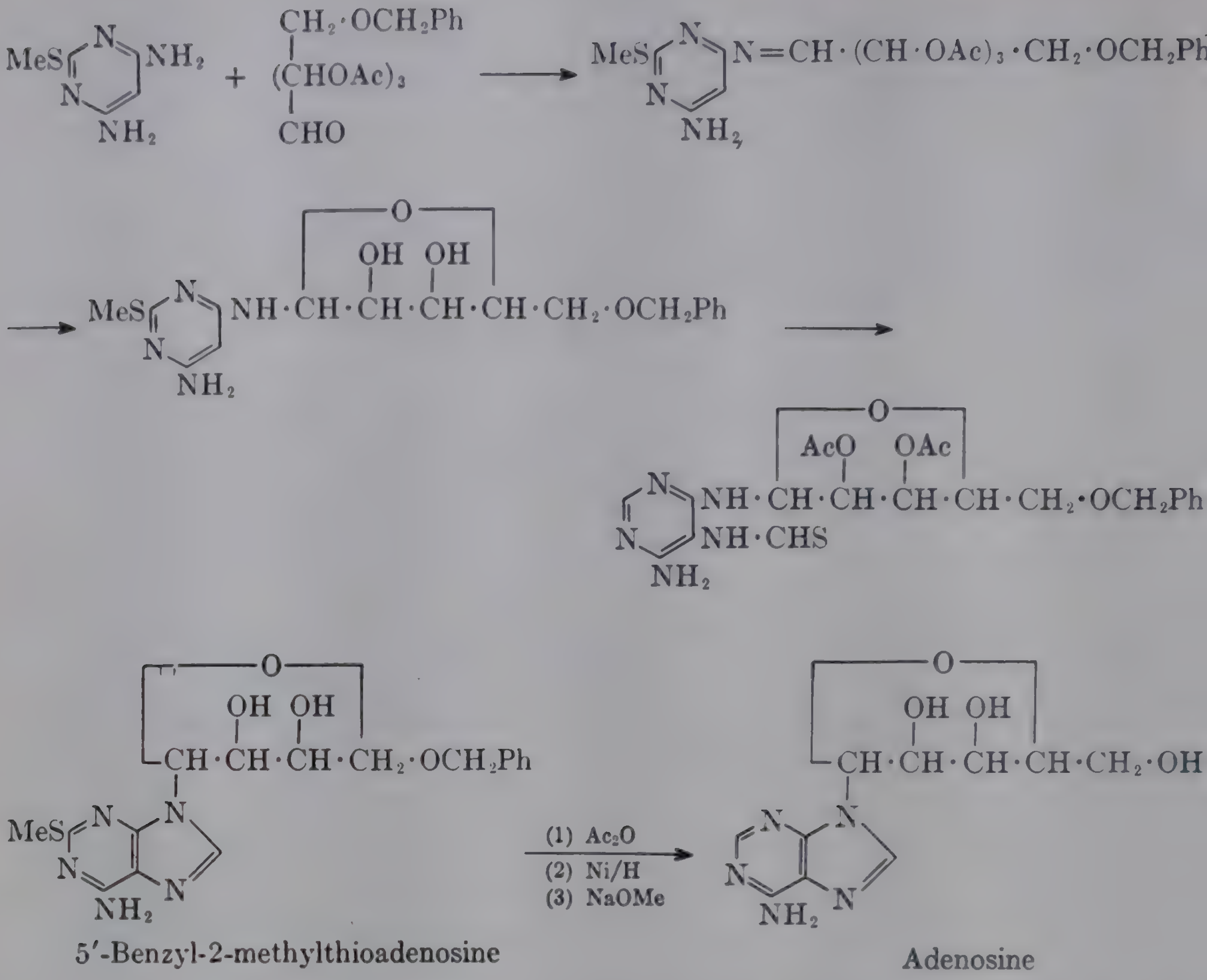
[76] G. A. Howard, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1945,** 556.

[77] K. J. M. Andrews, Nity Anand, A. R. Todd, and A. Topham, *J. Chem. Soc.* **1949,** 2490.

[78] G. A. Howard, G. W. Kenner, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1946,** 855.

[79] G. A. Howard, G. W. Kenner, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1946,** 861.

[80] G. W. Kenner, C. W. Taylor, and A. R. Todd, *J. Chem. Soc.* **1949,** 1620.



5′-Benzyl-2-methylthioadenosine

Adenosine

are obtained.[81, 82] However, acetyl groups may be used for protection, if at all stages deacetylation is avoided. In this way 9-D-galactofuranosyl-2-methylthioadenine has been prepared from 2,3,5,6-tetraacetyl-D-galactofuranose.[83]

Purine nucleosides may also be synthesized from iminazoles. This method is more or less restricted to the xanthine glycosides,[84, 85] but with that limitation is quite useful and has been applied to the synthesis of xanthosine itself.[86] Starting from an acetohalogeno-sugar and the silver derivative of 4,5-dicarbomethoxyiminazole a glycoside is obtained. This is converted to a diamide with simultaneous deacetylation by the action of ammonia, and cyclization to the purine is effected by potassium hypobromite in a modified Hofmann reaction.

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[81] G. W. Kenner, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1948,** 957.

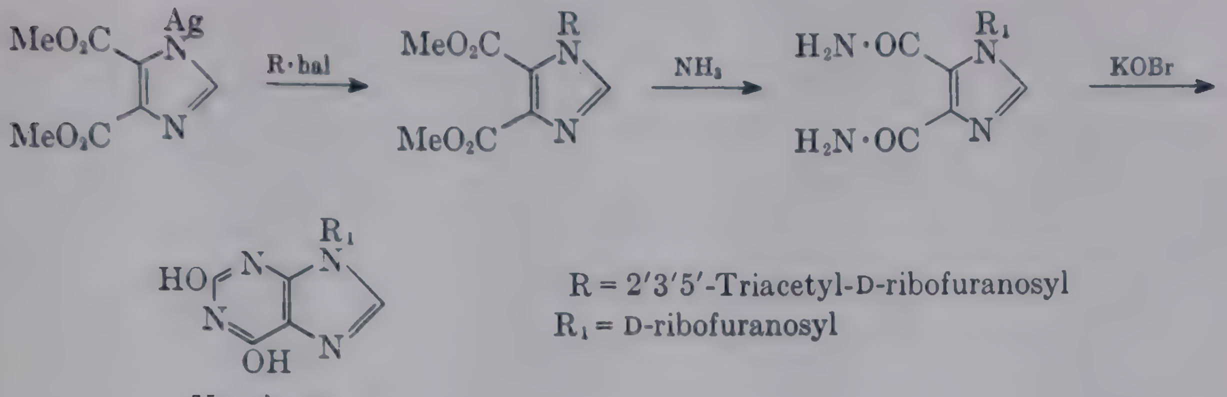
[82] G. W. Kenner, H. J. Rodda, and A. R. Todd, *J. Chem. Soc.* **1949,** 1613.

[83] K. J. M. Andrews, G. W. Kenner, and A. R. Todd, *J. Chem. Soc.* **1949,** 2302.

[84] R. A. Baxter and F. S. Spring, *J. Chem. Soc.* **1947,** 378.

[85] R. A. Baxter, A. C. McLean, and F. S. Spring, *J. Chem. Soc.* **1948,** 523.

[86] G. A. Howard, A. C. McLean, G. T. Newbold, F. S. Spring, and A. R. Todd, *J. Chem. Soc.* **1949,** 232.

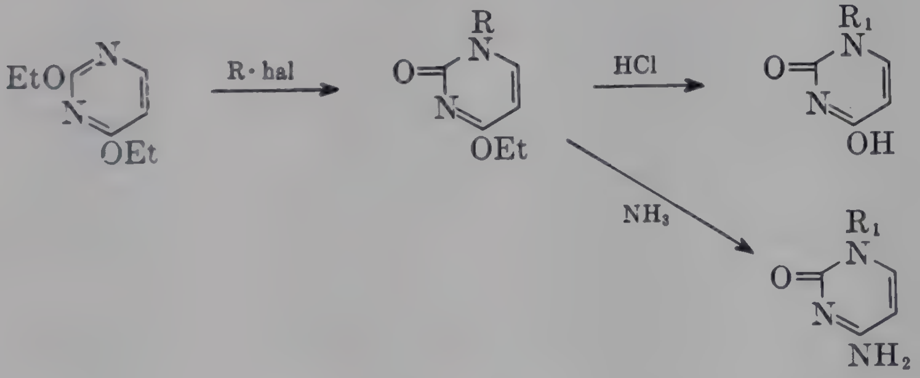


Xanthosine

R = 2′3′5′-Triacetyl-D-ribofuranosyl
R₁ = D-ribofuranosyl

b. *Pyrimidine Nucleosides*

Pyrimidine nucleosides have been synthesized from acetohalogeno-sugars and suitably substituted pyrimidines. When the pyrimidine employed bears substituents, such as hydroxyl, which are capable of prototropic change, the glycosyl residue becomes attached thereto and heterocyclic *N*-glycosides cannot be produced in this manner.[50, 52, 57] However, *N*-glycosides are obtained by the action of acetohalogeno-sugars on 2,6-dialkoxypyrimidines, in which tautomerization possibilities are excluded. This is an extension of the reaction observed between alkyl halides and 2,6-dialkoxypyrimidines whereby 3-alkyl-6-alkoxy-2-ketopyrimidines are formed.



Thus, interaction of acetobromoglucose and 2,6-diethoxypyrimidine gives an acetylglucoside which on hydrolysis with hydrogen chloride in methanol yields 3-D-glucosyluracil. Alternatively, by treatment of the acetylated glucoside with ammonia, 3-D-glucosylcytosine is formed.[87, 88] Several structural analogues have been prepared by this method.[89, 90] Starting from acetobromoribofuranose and 2,6-diethoxypyrimidine the natural nucleo-

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[87] G. E. Hilbert and T. B. Johnson, *J. Am. Chem. Soc.* **52,** 4489 (1930).

[88] G. E. Hilbert and E. F. Jansen, *J. Am. Chem. Soc.* **58,** 60 (1936).

[89] G. E. Hilbert, *J. Biol. Chem.* **117,** 331 (1937).

[90] G. E. Hilbert and C. E. Rist, *J. Biol. Chem.* **117,** 371 (1937).

TABLE I

PROPERTIES OF NUCLEOSIDES

Nucleoside	M.p., °C.	$[\alpha]_D$ (H_2O)	p*K*	Derivatives
Adenosine	229	−60.0° [93]	amino 3.3[93, 94] sugar 12.5	picrate, m.p. 180–185° (dec.)
Guanosine	237–240	−60.52° (alkali)	amino 1.6[94] hydroxyl 9.16 sugar 12.5	2′,3′,5′-triacetylguanosine, m.p. 226° [95]
Uridine	165	4.0° [93] −6.0° alkali	hydroxyl 9.17[94] sugar 12.5	5-bromo, m.p. 181–184°;[11] 5-hydroxy, m.p. 222–223°
Cytidine	220–230	29.63°	amino 4.22[93, 94] sugar 12.5	picrate, m.p. 185–187°; sulfate, m.p. 233°; hydrochloride, m.p. 218°; nitrate, m.p. 197°
Inosine	218	−47.7° [8]	hydroxyl 8.75[93, 94] sugar 12.5	2′,3′,5′-triacetylinosine, m.p. 236°
Xanthosine		−51.2° (alkali)		
Adenine deoxyriboside	189–190[96]			
Hypoxanthine deoxyriboside	not sharp	−21.0°		
Guanine deoxyriboside	not sharp	−37.5°		
Thymidine	182–183 186	32.5° (in alkali)		
Cytosine deoxyriboside		40.0°		hydrochloride, m.p. 161–164° (dec.)[96]
Uracil deoxyriboside	163[3]			
5-Methylcytosine deoxyriboside				picrate, m.p. 175–178°, is converted into thymidine by nitrous acid[96a]

sides uridine and cytidine are obtained.[91] Thymine nucleosides are synthesized in a similar manner from 2,6-diethoxy-5-methylpyrimidine.[92]

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[91] G. A. Howard, B. Lythgoe, and A. R. Todd, *J. Chem. Soc.* **1947,** 1052.

[92] D. W. Visser, I. Goodmann, and K. Dittmer, *J. Am. Chem. Soc.* **70,** 1926 (1948).

[93] P. A. Levene, *J. Biol. Chem.* **41,** 483 (1920); cf. P. A. Levene, H. S. Simms, and L. W. Bass, *ibid.* **70,** 243 (1926).

[94] P. A. Levene and H. S. Simms, *J. Biol. Chem.* **65,** 519 (1925).

[95] H. Steudel and R. Freise, *Z. physiol. Chem.* **120,** 126 (1922).

[96] W. Andersen, C. A. Dekker, and A. R. Todd, *J. Chem. Soc.* **1952,** 2721.

[96a] C. A. Dekker and D. T. Elmore, *J. Chem. Soc.* **1951,** 2864.

4. General Properties of Nucleosides

The nucleosides in general are colorless, crystalline substances with rather high melting points. They vary somewhat in their solubility in water, but most representatives are readily soluble in hot, considerably less soluble in cold water. The pyrimidine nucleosides uridine and cytidine are more soluble in water than are the purine nucleosides. Both pyrimidine and purine nucleosides are insoluble in the more common organic solvents. Nucleosides containing amino groups (adenosine, cytidine) are rather weak bases, while those containing hydroxyl groups on the heterocyclic nucleus (xanthosine, inosine, uridine) are weak acids.

Physical properties and useful derivatives of some of the better known natural nucleosides are listed in Table I.

5. Miscellaneous Nucleosides

The nucleosides inosine, xanthosine, hypoxanthine deoxyriboside, and uracil deoxyriboside which have been described in the previous sections are thought to be absent from the native nucleic acids, but frequently arise during isolation, or during hydrolysis of the isolated macromolecule to its component nucleosides. In addition to these, some nucleosides which have been isolated from natural sources are neither components nor degradation products of nucleic acids. These are discussed below.

a. Adenine Thiomethyl Pentoside (5′-deoxy-5′-methylthioadenosine)

This nucleoside was first isolated from yeast,[97] but is probably present in the tissues of many animals. On hydrolysis it gives adenine and a sulfur-containing sugar, 5-deoxy-5-methylthioribose. The structure of the aldopentose follows from its reduction to the pentitol, periodate titration, and direct comparison with synthetic 5-deoxy-5-methylthiopentose derivatives.[98-101] The ultraviolet absorption spectrum of the nucleoside supports a 9-glycoside structure.[102] With nitrous acid it gives "hypoxanthine thiomethyl pentoside,"[103] the structure of which has been proved by synthesis.[104, 105] This synthesis starts from 2′,3′-isopropylidene-5′-*p*-toluene sulfonylinosine,[17] from which is prepared 2′,3′-isopropylidene-5′-methylthio-5′-deoxyinosine. The isopropylidene residue may be removed by cautious acid hydrolysis to give 5′-deoxy-5′-methylthioinosine, identica with "hypoxanthine thiomethyl pentoside."

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[97] J. A. Mandel and E. K. Dunham, *J. Biol. Chem.* **11,** 85 (1912).

[98] P. A. Levene and H. Sobotka, *J. Biol. Chem.* **65,** 551 (1925).

[99] G. Wendt, *Z. physiol. Chem.* **272,** 152 (1942).

[100] K. Satoh and K. Makino, *Nature,* **165,** 769 (1950).

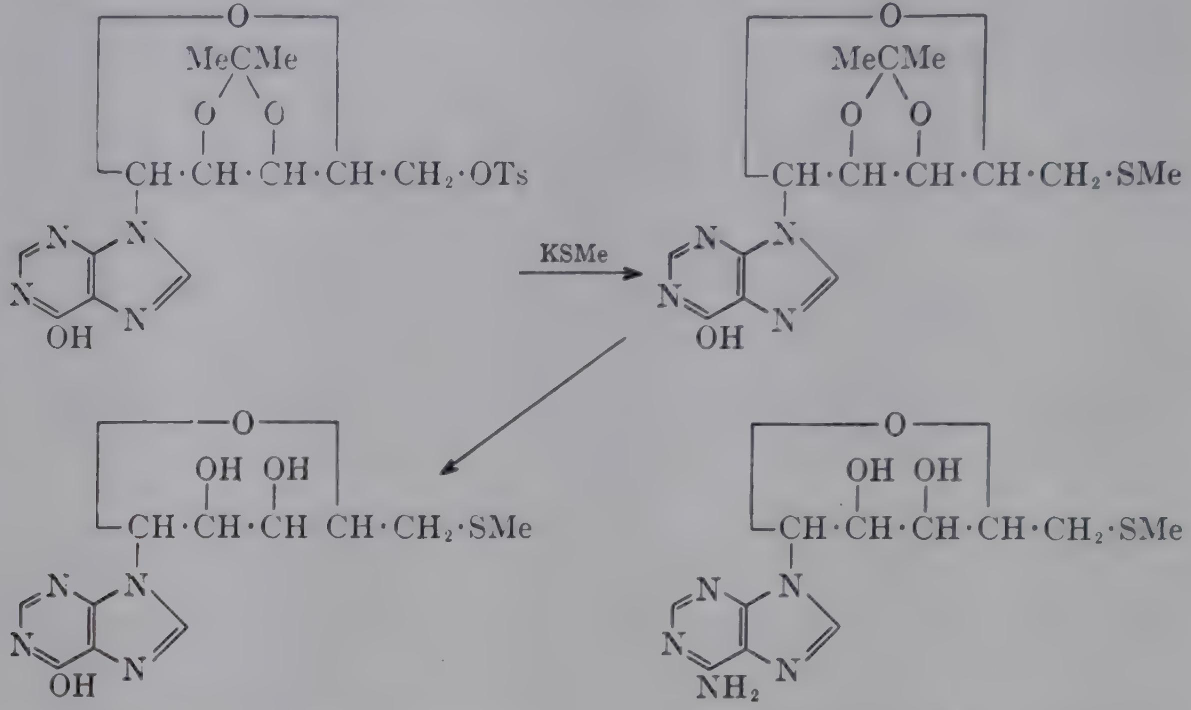
[101] F. Weygand, O. Trauth, and R. Löwenfeld, *Ber.* **83,** 563 (1950).

[102] R. Falconer and J. M. Gulland, *J. Chem. Soc.* **1937,** 1912.

[103] R. Kuhn and K. Henkel, *Z. physiol. Chem.* **269,** 41 (1941).

[104] J. Baddiley, *J. Chem. Soc.* **1951,** 1348.

[105] K. Satoh and K. Makino, *Nature* **167,** 238 (1951).

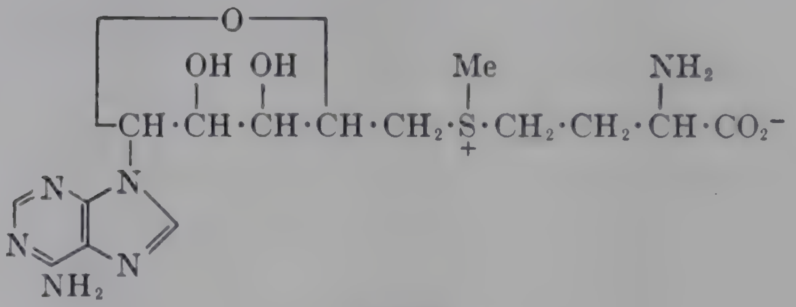


Hypoxanthine thiomethyl pentoside

Adenine thiomethyl pentoside

This synthesis establishes the β-configuration at the glycosidic center. Adenine thiomethyl pentoside itself is synthesized in poor yield by a similar series of reactions upon 2′,3′-isopropylidene-5′-*p*-toluenesulfonyladenosine.[104-106]

It is now known[107] that adenine thiomethyl pentoside arises in Nature by decomposition of the transmethylation intermediate "active methionine." This intermediate has the structure[107, 108] shown as XVIII.



XVIII
"Active methionine"

b. Crotonoside (9-β-D-ribofuranosylisoguanine)

This was first isolated from the bean *Croton tiglium*. It is isomeric with guanosine and gives on acid hydrolysis D-ribose and isoguanine.[109] Its ab-

[106] F. Weygand and O. Trauth, *Ber.* **84,** 633 (1951).

[107] J. Baddiley, G. L. Cantoni, and G. A. Jamieson, *J. Chem. Soc.*, **1953,** 2662.

[108] G. L. Cantoni, *J. Am. Chem. Soc.* **74,** 2942 (1952).

[109] E. Cherbuliez and K. Bernhard, *Helv. Chim. Acta* **15,** 464 (1932).

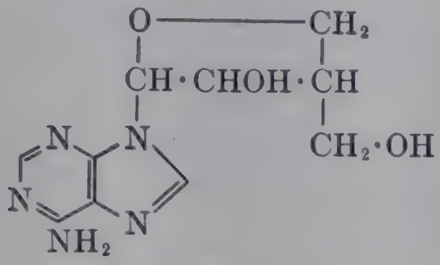
sorption spectrum indicates a 9-glycoside[110] and with nitrous acid it is converted into xanthosine. The formula shown on page 152 is confirmed by synthesis.

c. *Uric Acid Riboside*

The nucleoside is present in beef blood. It is hydrolyzed in acid to D-ribose and uric acid[111] and is probably a 9-glycoside.[112]

d. *Cordycepin* (*9-cordyceposyladenine*)

The mould *Cordyceps militaris* (Linn.) contains an antibiotic, cordycepin,[113] which gives on hydrolysis adenine and a deoxyaldopentose, cordycepose. Cordycepin is probably a 9-glycoside with the structure XIX.[114]



XIX
Cordycepin

e. *Spongothymidine*

This occurs in the sponge *Cryptotethia*. It yields thymine on hydrolysis but the sugar component has not been fully identified.[115, 115a]

f. *Orotidine*

A mutant of the mould *Neurospora crassa* contains a glycoside of orotic acid. On hydrolysis it yields ribose and orotic acid.[116]

g. *Vicine*

Vetch meal contains a pyrimidine glycoside, vicine.[117] It can be hydrolyzed by acids to D-glucose and the pyrimidine divicine.[118] Evidence for its structure is not entirely satisfactory. [Cf. *Bendich*, Chapter 3.]

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[110] R. Falconer and J. M. Gulland. *J. Chem. Soc.* **1939,** 1784.

[111] A. R. Davis, E. B. Newton, and S. R. Benedict, *J. Biol. Chem.* **54,** 595 (1922).

[112] R. Falconer and J. M. Gulland, *J. Chem. Soc.* **1939,** 1369.

[113] K. G. Cunningham, S. A. Hutchinson, W. Manson, and F. S. Spring, *J. Chem. Soc.* **1951,** 2299.

[114] H. R. Bentley, K. G. Cunningham, and F. S. Spring, *J. Chem. Soc.* **1951,** 2301.

[115] W. Bergmann and R. J. Feeney, *J. Am. Chem. Soc.* **72,** 2809 (1950).

[115a] According to a private communication from Drs. W. Bergmann and D. Burke, Yale University, spongothymidine is an arabinoside [THE EDITORS].

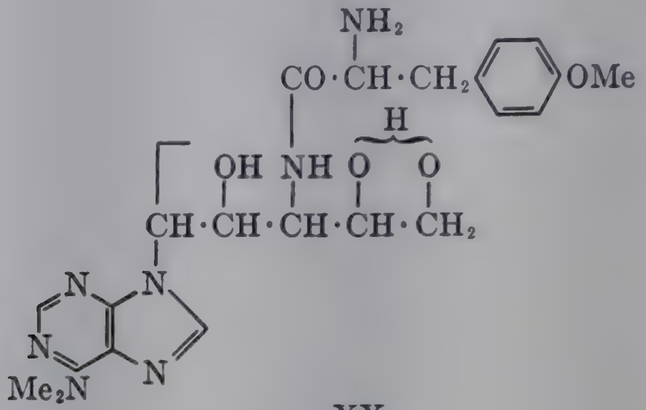
[116] A. M. Michelson, W. Drell, and H. K. Mitchell, *Proc. Natl. Acad. Sci. U. S.* **37,** 396 (1951).

[117] H. Ritthausen, *Ber.* **29,** 894, 2108 (1896).

[118] P. A. Levene, *J. Biol. Chem.* **18,** 305 (1914).

h. *Puromycin*

An antibiotic, puromycin, is produced by *Streptomyces alboniger*. On alcoholysis it yields a 6-dimethylaminopurine, *O*-methyl-L-tyrosine and D-3-amino-3-deoxyribose. Its ultraviolet absorption spectrum indicates that the carbohydrate residue is attached to the purine at position 9. Two hydroxyls and an amino group are present and it does not consume periodate. These properties are consistent with the partial formula XX.[118a] It is not known whether the carbohydrate residue is in the furanose or pyranose configuration.



XX
Puromycin

II. Nucleotides

1. Introduction

Nucleotides are the phosphoric acid esters of nucleosides. They may be prepared by chemical or enzymic hydrolysis of nucleic acids provided that the conditions chosen do not effect hydrolysis of phosphomonoester groups. Nucleotides are also formed during hydrolysis of certain coenzymes, but nucleic acids are the most convenient source for these substances. The first nucleotide to be isolated, inosinic acid,[119] is probably exceptional in this respect. It was obtained from meat by fairly direct methods of extraction and purification and is believed to be an artifact arising through deamination of "muscle adenylic acid" (adenosine-5′-phosphate), which occurs in the free state in muscle tissues.[120]

Ribonucleotides may be obtained from ribonucleic acid by gentle chemical or enzymic hydrolysis. Enzymic hydrolysis[121] is incomplete, however, and chemical methods are generally employed for preparative purposes. Hydrolysis of yeast ribonucleic acid with dilute ammonia gives the nucleotides known as yeast adenylic acid, guanylic, uridylic, and cytidylic acids.[122, 123] Dilute alkali effects a nearly quantitative conversion to the nucleotide mixture.[123a] Improved methods have been introduced from time to time[124] and cautious acid hydrolysis is convenient when only the more stable pyrimidine nucleotides are required.[125-127] However, recent investigations on these nucleotides using refined methods of ion-exchange and paper chromatography as well as selective fractional crystallization procedures, show that they are not homogeneous and consequently the older isolation techniques are no longer used. [Cf. Chapters 5–7, 11, 12.] By suitable combination of ion-exchange and paper chromatography it can be shown that the nucleotides produced by hydrolysis of ribonucleic acid are mixtures of isomers. Thus, yeast adenylic acid is a mixture of the two isomers adenylic acid *a* and adenylic acid *b*. Similarly, guanylic, cytidylic, and uridylic acids are all mixtures of *a* and *b* isomers.[128-132] Certain of the isomers may be separated from each other by fractional crystallization of cyclohexylamine and brucine salts.[133, 134] It should be explained that the terms *a* and *b* were originally assigned arbitrarily and did not necessarily signify any structural correlation between the different members. As will be seen later, it is now known that cytidylic acid *a* is structurally related to uridylic acid *a*, similarly cytidylic acid *b* is related to uridylic acid *b*. The isomerism in all these nucleotides involves the position of the phosphate residue on C-2′, or C-3′, in the respective nucleosides.

[118a] C. W. Waller, P. W. Fryth, B. L. Hutchings, and J. H. Williams. *J. Am. Chem. Soc.* **75,** 2025 (1953).

[119] J. von Liebig, *Ann.* **62,** 257 (1847).

[120] G. Embden and M. Zimmermann, *Z. physiol. Chem.* **167,** 137 (1927).

[121] H. S. Loring and F. H. Carpenter. *J. Biol. Chem.* **150,** 381 (1943).

Nucleoside-5′-phosphates may also be obtained from ribonucleic acid. For reasons discussed in Chapter 12, chemical hydrolysis cannot give these nucleotides. However, ribonuclease hydrolysis under conditions which do not permit 5′-phosphatases to operate, followed by ion-exchange separation of the products, yields 5′-phosphates.[135] [Cf. *Cohn*, Chapter 6; *Schmidt*, Chapter 15.]

[122] P. A. Levene, *J. Biol. Chem.* **33,** 425 (1918).
[123] P. A. Levene, *J. Biol. Chem.* **40,** 415 (1919).
[123a] E. Chargaff, B. Magasanik, E. Vischer, C. Green, R. Doniger, and D. Elson, *J. Biol. Chem.* **186,** 51 (1950).
[124] H. S. Loring, P. M. Roll, and J. G. Pierce, *J. Biol. Chem.* **174,** 729 (1948).
[125] P. A. Levene and W. A. Jacobs, *Ber.* **44,** 1027 (1911).
[126] S. J. Thannhauser and G. Dorfmüller, *Z. physiol. Chem.* **104,** 65 (1919).
[127] H. Bredereck and F. Richter, *Ber.* **71,** 718 (1938).
[128] W. E. Cohn, *Science,* **109,** 377 (1949).
[129] W. E. Cohn, *J. Am. Chem. Soc.* **71,** 2275 (1949).
[130] W. E. Cohn, *J. Am. Chem. Soc.* **72,** 2811 (1950).
[131] W. E. Cohn and C. E. Carter, *J. Am. Chem. Soc.* **72,** 2606 (1950).
[132] C. E. Carter, *J. Am. Chem. Soc.* **72,** 1466 (1950).
[133] H. S. Loring and N. G. Luthy, *J. Am. Chem. Soc.* **73,** 4215 (1951).
[134] P. Reichard, Y. Takenaka, and H. S. Loring, *J. Biol. Chem.* **198,** 599 (1952).
[135] W. E. Cohn and E. Volkin, *Nature* **167,** 483 (1951).

Ribonuclease digestion, or very mild alkaline hydrolysis of ribonucleic acid, gives the four possible cyclic nucleoside-2′,3′-hydrogen phosphates, together with other products.[136] These are artifacts, cyclic phosphate structures not being present in nucleic acids, but their formation is significant in connection with the structures proposed for ribonucleic acid (see Chapter 12). Ribonuclease digestion of ribonucleic acid also yields a number of di- and trinucleotides, some of which contain cyclic phosphate structures.[137, 138]

Pyrimidine deoxyribonucleotides are produced by mild acid hydrolysis of deoxyribonucleic acid.[139-145] Both 5′-phosphates and 3′,5′-diphosphates are obtained. The purine deoxyribonucleotides are very sensitive to acids and cannot be prepared by chemical hydrolysis of the parent nucleic acid. However, these substances are obtained from the nucleic acid by using an intestinal deoxyribonuclease in the presence of sodium arsenate, thereby inhibiting the action of an accompanying nucleotidase.[146-149] Recent methods for the isolation of deoxyribonucleotides employ ion-exchange resins.[145, 150, 151]

2. STRUCTURE OF NUCLEOTIDES

As the nucleotides may differ from each other not only in the nature of the pyrimidine and purine bases attached to either of the sugars D-ribose and D-2-deoxyribose, but also in the location of the phosphoric ester linkage, they are best considered individually.

a. Inosine-5′-phosphate (muscle inosinic acid) (XXI)

This nucleotide is a phosphoric ester containing the components hypoxanthine[152] and D-ribose.[7-9] On neutral hydrolysis it gives phosphoric acid and inosine; consequently it is a monophosphate of inosine in which the phosphate residue is located at the 2′,3′- or 5′-position on the sugar chain.[153] The ribose phosphate produced by acid hydrolysis must be ribose-5-phosphate since it is oxidized with nitric acid to a ribonic acid phosphate and not a *ribo*-trihydroxyglutaric acid phosphate.[9]

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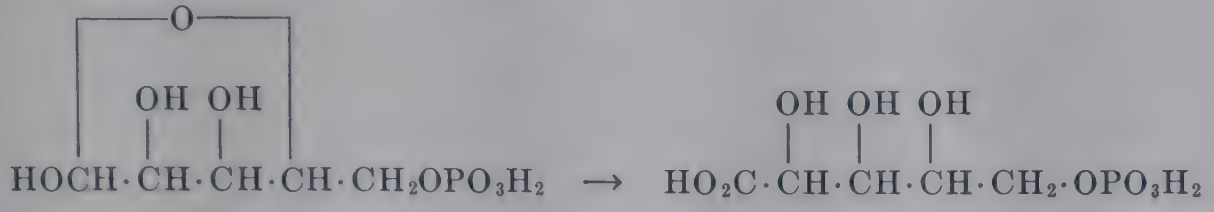
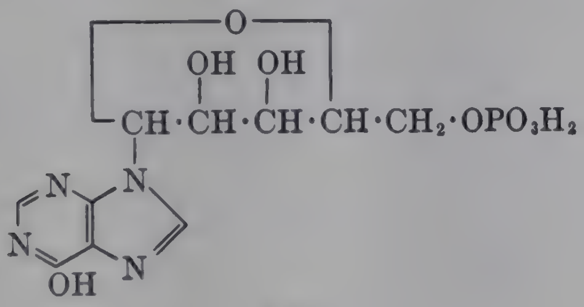
[136] R. Markham and J. D. Smith, *Biochem. J.* **52,** 552 (1952).
[137] R. Markham and J. D. Smith, *Biochem. J.* **52,** 558 (1952).
[138] R. Markham and J. D. Smith, *Biochem. J.* **52,** 565 (1952).
[139] P. A. Levene and H. Mandel, *Ber.* **41,** 1905 (1908).
[140] P. A. Levene and W. A. Jacobs, *J. Biol. Chem.* **12,** 411 (1912).
[141] P. A. Levene, *J. Biol. Chem.* **48,** 119 (1921).
[142] S. J. Thannhauser and B. Ottenstein, *Z. physiol. Chem.* **114,** 39 (1921).
[143] S. J. Thannhauser and G. Blanco, *Z. physiol. Chem.* **161,** 116 (1926).
[144] P. A. Levene, *J. Biol. Chem.* **126,** 63 (1938).
[145] C. A. Dekker, A. M. Michelson, and A. R. Todd, *J. Chem. Soc.* **1953,** 947.
[146] W. Klein, *Z. physiol. Chem.* **218,** 164 (1933).
[147] W. Klein and S. J. Thannhauser, *Z. physiol. Chem.* **218,** 173 (1933).
[148] W. Klein and S. J. Thannhauser, *Z. physiol. Chem.* **224,** 252 (1934).
[149] W. Klein and S. J. Thannhauser, *Z. physiol. Chem.* **231,** 96 (1935).
[150] E. Volkin, J. X. Khym, and W. E. Cohn, *J. Am. Chem. Soc.* **73,** 1533 (1951).
[151] R. L. Sinsheimer and J. F. Koerner, *Science* **114,** 42 (1951).
[152] F. Haiser, *Monatsh.* **16,** 190 (1895).



$$\mathrm{HOCH\cdot \overset{OH}{CH}\cdot \overset{OH}{CH}\cdot CH\cdot CH_2OPO_3H_2 \longrightarrow HO_2C\cdot \overset{OH}{CH}\cdot \overset{OH}{CH}\cdot \overset{OH}{CH}\cdot CH_2\cdot OPO_3H_2}$$

D-Ribose-5-phosphate D-Ribonic acid-5-phosphate

The structure of ribonic acid-5-phosphate is confirmed by its slow conversion into a γ-lactore.[154, 155] Also, the sugar phosphate itself is converted into a methylribofuranoside-5-phosphate rather than a mixture of furanoside and pyranoside[156] and is reduced to optically active ribitol-5-phosphate. Full proof for the structure of ribose-5-phosphate is given by its synthesis from methyl 2,3-isopropylideneribofuranoside. Phosphorylation with phosphoryl chloride, then acid hydrolysis, gives the 5-phosphate.[157] Better yields are obtained by phosphorylation with dibenzyl phosphorochloridate, then hydrogenolysis and acid hydrolysis.[158]



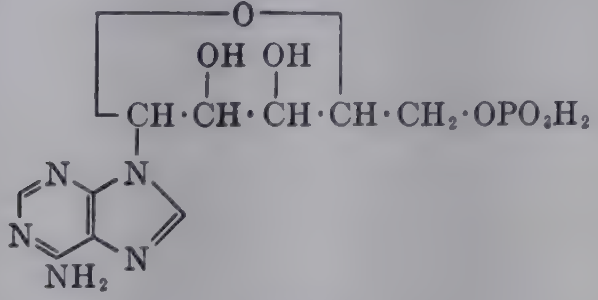
XXI
Muscle inosinic acid

b. Adenosine-5′-phosphate (muscle adenylic acid) (XXII)

As its name implies, this nucleotide was first isolated from muscle,[120] but it is now known to occur as a structural unit of ribonucleic acid and of several coenzymes. Since it can be deaminated to inosine-5′-phosphate by an enzyme present in muscle tissue,[159] it must bear the same relationship to that nucleotide as does adenosine to inosine, i.e., it must be adenosine-5′-phosphate. Furthermore, the two nucleotides liberate phosphoric acid at

[153] P. A. Levene and W. A. Jacobs, *Ber.* **42,** 335 (1909).
[154] P. A. Levene and H. S. Simms, *J. Biol. Chem.* **65,** 31 (1925).
[155] P. A. Levene and T. Mori, *J. Biol. Chem.* **81,** 215 (1929).
[156] P. A. Levene, S. A. Harris, and E. T. Stiller, *J. Biol. Chem.* **105,** 153 (1934).
[157] P. A. Levene and E. T. Stiller, *J. Biol. Chem.* **104,** 299 (1934).
[158] A. M. Michelson and A. R. Todd, *J. Chem. Soc.* **1949,** 2476.
[159] G. Schmidt, *Z. physiol. Chem.* **179,** 243 (1928).

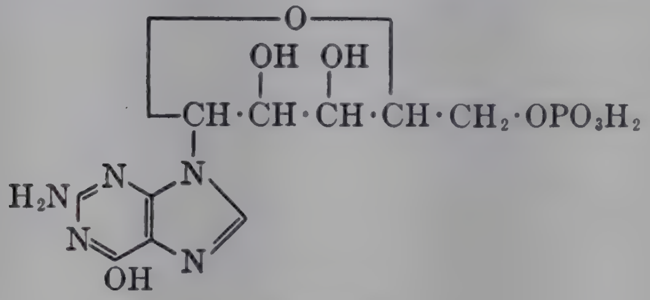
the same rate during alkaline hydrolysis[169] and adenosine-5′-phosphat gives adenosine when dephosphorylated by alkaline phosphatase.[161] Th presence of an unsubstituted *cis*-1,2-glycol structure in adenosine-5′-phosphate is indicated by the ready formation of its boric acid complex.[162]



XXII
Muscle adenylic acid

c. *Guanosine-5′-phosphate (XXIII)*

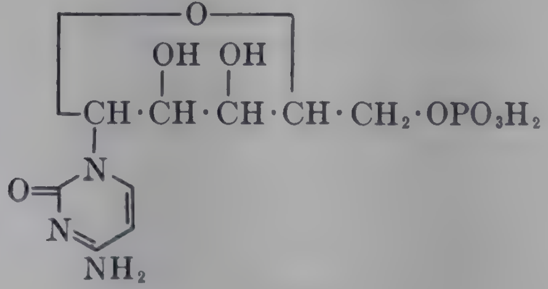
It is now known that guanosine-5′-phosphate is produced during enzymi hydrolysis of ribonucleic acid.[135] Although it is converted into guanosin through the action of a 5′-phosphatase, it is different from the isomeri guanylic acids *a* and *b*. The structure of this nucleotide was established b direct comparison with synthetic guanosine-5′-phosphate.



XXIII
Guanosine-5′-phosphate

d. *Cytidine-5′-phosphate (XXIV)*

Enzymic hydrolysates of ribonucleic acid may also contain cytidine-5′ phosphate.[135] Similar structural considerations apply to this nucleotide a



XXIV
Cytidine-5′-phosphate

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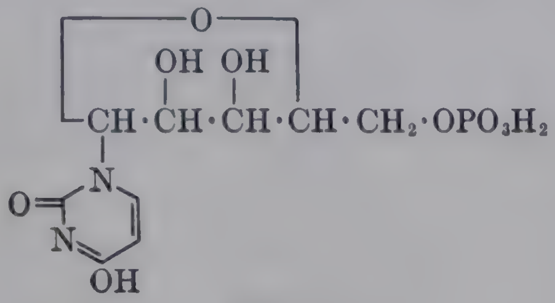
[160] G. Embden and G. Schmidt, *Z. physiol. Chem.* **181,** 130 (1929).
[161] J. M. Gulland and E. R. Holiday, *J. Chem. Soc.* **1936,** 765.
[162] R. Klimek and J. K. Parnas, *Biochem. Z.* **292,** 356 (1937).

for guanosine-5′-phosphate, and its identity with synthetic cytidine-5′-phosphate has been established.

e. *Uridine-5′-phosphate (XXV)*

Although uridine-5′-phosphate may be formed by enzymic hydrolysis of ribonucleic acid[135] it is also present as a structural unit in the uridine diphosphate coenzymes. It is hydrolyzed either enzymically or chemically to uridine and consumes 1 mol. periodate, so it must be a 5′-phosphate.[163] It is identical with synthetic uridine-5′-phosphate.



XXV
Uridine-5′-phosphate

f Adenylic Acids a *and* b

When ribonucleic acid is subjected to cautious hydrolysis, a material known as "yeast adenylic acid" is obtained, together with other nucleotides and nucleosides.[122, 164, 165] The early workers considered that this was a single substance, but recent work has shown that two isomeric adenylic acids (*a* and *b*) are formed by hydrolysis of ribonucleic acid, and it is not quite certain which isomer was under investigation at each stage of the initial structural work. In fact, it is probable that both isomers were present on some occasions. For these reasons, and also because of the ready interconversion of the isomers under mild acid conditions, the early work on the location of the phosphate group in yeast adenylic acid is unreliable. However, it was clear that yeast adenylic acid is a monophosphate (or mixture of monophosphates) of adenosine, since it yields adenosine and phosphoric acid in equal amounts on hydrolysis in ammonia.[165] Furthermore, the phosphate group must be situated on the sugar residue, since after treatment with nitrous acid it gives an inosine phosphate which hydrolyzes readily to hypoxanthine and a ribose phosphate (or mixture of ribose phosphates).[166] This "ribose phosphate" differs from ribose-5-phosphate. It would appear, then, that yeast adenylic acid is adenosine-2′- or adenosine-3′-phosphate (XXVI, XXVII). The ribose phosphate obtained from yeast adenylic acid

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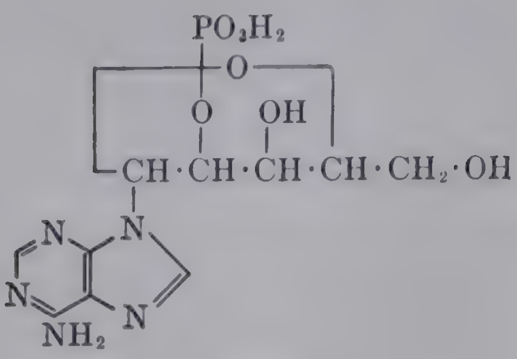
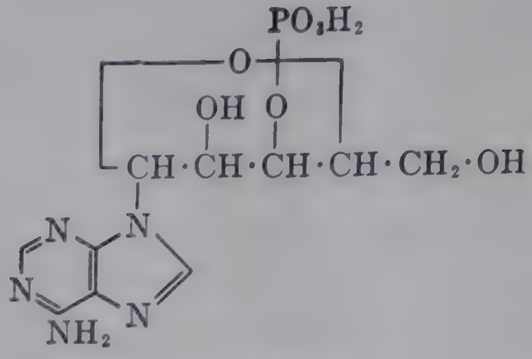
[163] A. C. Paladini and L. F. Leloir, *Biochem. J.* **51,** 426 (1952).
[164] W. Jones and R. P. Kennedy, *J. Pharmacol.* **13,** 45 (1919).
[165] S. J. Thannhauser, *Z. physiol. Chem.* **107,** 157 (1919).
[166] P. A. Levene and S. A. Harris, *J. Biol. Chem.* **101,** 419 (1933).

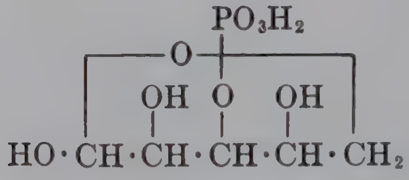


XXVI
Adenosine-2′-phosphate

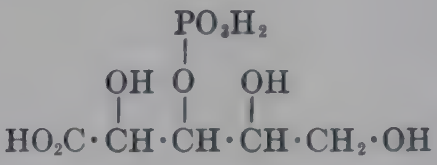


XXVII
Adenosine-3′-phosphate

was believed to be identical with that from guanylic acid and gave on oxida tion a ribonic acid phosphate which differed in rate of lactonization an hydrolysis from ribonic acid-5-phosphate.[167] Furthermore, it was reduce catalytically to an optically inactive ribitol phosphate.[168] If the yeas adenylic acid in the first place was a single substance, and if no migratio of phosphate groups had occurred during subsequent hydrolysis and re duction, then the above transformations would indicate that the nucleotid is adenosine-3′-phosphate yielding ribose-3-phosphate, and ribitol-3-phos phate.

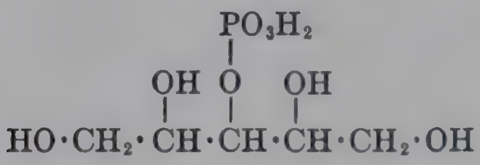


Ribose-3-phosphate



$$HO_2C\cdot\underset{}{\overset{OH}{CH}}\cdot\overset{O\cdot PO_3H_2}{CH}\cdot\overset{OH}{CH}\cdot CH_2\cdot OH$$

Ribonic acid-3-phosphate



$$HO\cdot CH_2\cdot\overset{OH}{CH}\cdot\overset{O\cdot PO_3H_2}{CH}\cdot\overset{OH}{CH}\cdot CH_2\cdot OH$$

Ribitol-3-phosphate

Since it is now known that both *a* and *b* forms of adenylic acid are produce by hydrolysis of ribonucleic acid[169] and also since it has been shown tha these acids are rapidly interconvertible under mild acid conditions,[170, 1 the structural investigations on yeast adenylic acid are of little value.

Adenylic acids *a* and *b* are both dephosphorylated enzymically to adeno sine,[132, 169] are stable to periodate oxidation,[171] and are relatively stable t alkali. These properties agree with those expected for adenosine-2′- an

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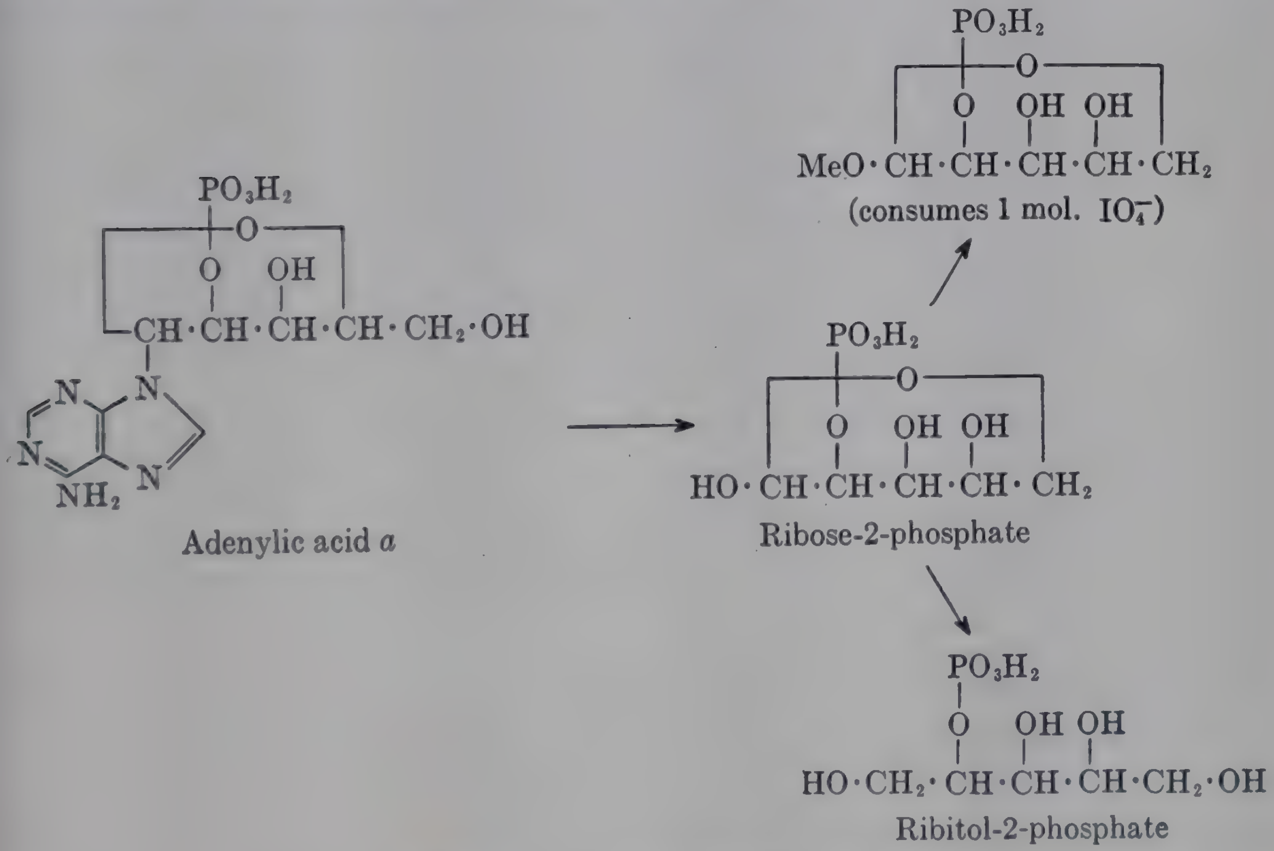
[167] P. A. Levene and S. A. Harris, *J. Biol. Chem.* **95,** 755 (1932).
[168] P. A. Levene and S. A. Harris, *J. Biol. Chem.* **98,** 9 (1932).
[169] C. E. Carter and W. E. Cohn, *Federation Proc.* **8,** 190 (1949).
[170] D. M. Brown and A. R. Todd. *J. Chem. Soc.* **1952,** 44.
[171] D. M. Brown, L. J. Haynes, and A. R. Todd, *J. Chem. Soc.* **1950,** 2299.

3′-phosphates[132, 172] and strong support for this view is furnished by the synthesis of the two isomers by phosphorylation of 5′-trityladenosine with dibenzyl phosphorochloridate, followed by hydrogenolysis of protecting groups[170] (see p. 174). Also, the ready cyclization of adenylic acids *a* and *b* to the same adenosine-2′,3′-hydrogen phosphate[173] can only be explained in this way. It has been shown recently[174] that adenylic acid *a* may be hydrolyzed by short boiling in water with an ion-exchange resin (sulfonic acid type) to ribose-2-phosphate. Under these conditions isomerization of nucleotides and ribose phosphates does not occur to any significant extent. After similar treatment adenylic acid *b* gives ribose-3-phosphate. The structure of the ribose phosphates follows from periodate oxidation of their methyl glycosides and reduction to ribitol phosphates. Ribose-3-phosphate is reduced to an optically inactive ribitol-3-phosphate, whereas the 2-phosphate gives an optically active substance, the activity of which may be enhanced by formation of a boric acid complex. It follows that adenylic acid *a* is adenosine-2′-phosphate and adenylic acid *b* is adenosine-3′-phosphate.

Adenosine-2′,5′-diphosphate is obtained by enzymic hydrolysis of triphosphopyridine nucleotide (p. 182) and the 3′,5′-diphosphate is produced in a similar manner from coenzyme A (p. 186).



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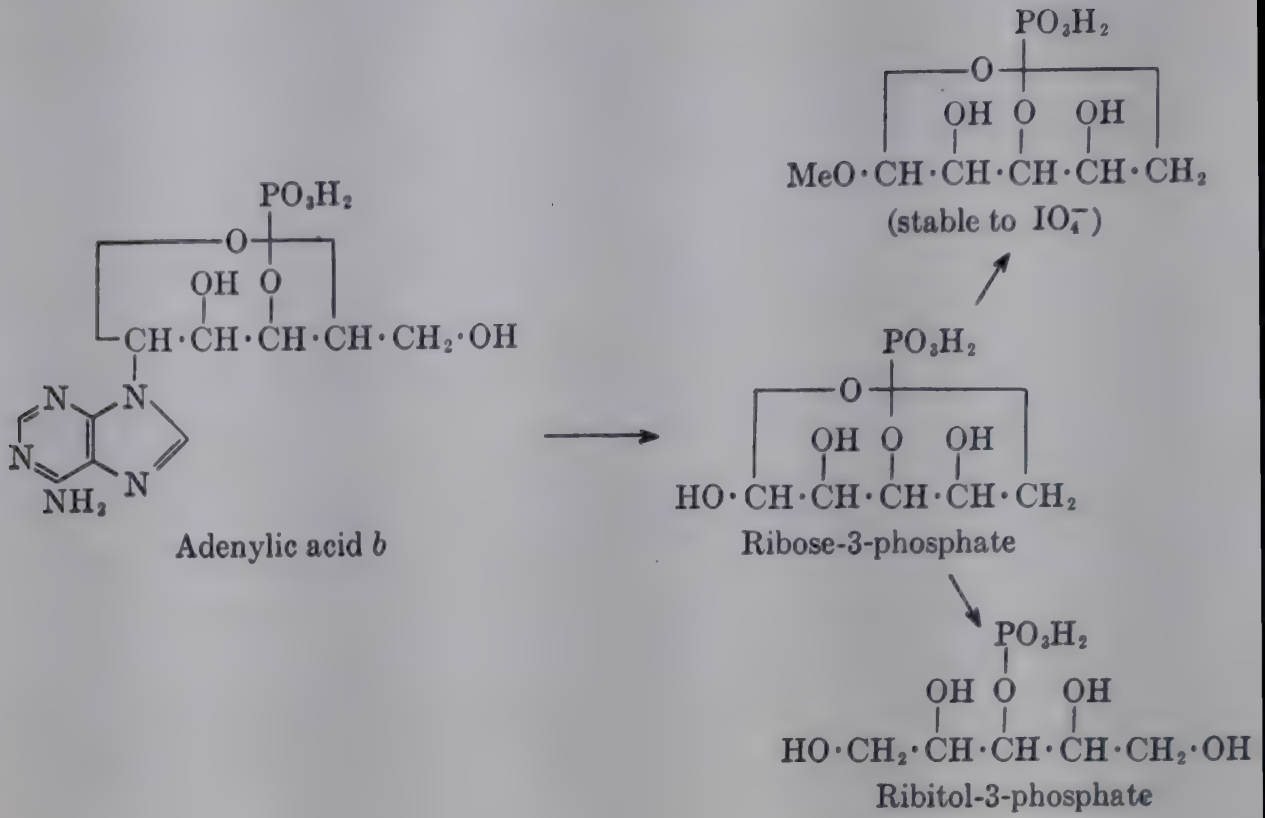
[172] D. M. Brown and A. R. Todd, *J. Chem. Soc.* **1952,** 52.

[173] D. M. Brown, D. I. Magrath, and A. R. Todd, *J. Chem. Soc.* **1952,** 2708.

[174] J. X. Khym, D. G. Doherty, E. Volkin, and W. E. Cohn, *J. Am. Chem. Soc.* **75,** 1262 (1953).



Adenylic acid *b*

Ribose-3-phosphate

Ribitol-3-phosphate

g. Guanylic Acids a *and* b

A substance known as guanylic acid was first isolated in 1898.[175] Although at first there was much controversy concerning its occurrence and composition, its existence was fully established eventually by Steudel.[176] It can be isolated in crystalline form by hydrolysis of ribonucleic acid,[93, 177] and it was believed that guanylic acid from different sources was a single substance.[178] On treatment with phosphatase[20] or by alkaline hydrolysis[179-181] guanylic acid is converted into guanosine, and with nitrous acid it is deaminated to xanthylic acid.[167, 182, 183] It follows that the phosphate group is situated on the sugar residue of guanosine. The ribose phosphate formed on hydrolysis of xanthylic acid[167, 182] was alleged to be identical with that obtained from yeast adenylic acid, consequently guanylic acid was thought to be guanosine-3′-phosphate. However, it is now known that the guanylic acid obtained by hydrolysis of ribonucleic acid is a mixture of *a* and *b* isomers,[130, 184] and it is assumed, by analogy with the adenylic acids, that these are 2′- and 3′-phosphates, but not necessarily respectively (XXVIII, XXIX).

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[175] I. Bang, *Z. physiol. Chem.* **26,** 133 (1898).

[176] H. Steudel, *Z. physiol. Chem.* **53,** 539 (1907).

[177] P. A. Levene, *J. Biol. Chem.* **40,** 171 (1919).

[178] P. A. Levene and E. Jorpes, *J. Biol. Chem.* **81,** 575 (1929).

[179] P. A. Levene and W. A. Jacobs, *Ber.* **42,** 2469 (1909).

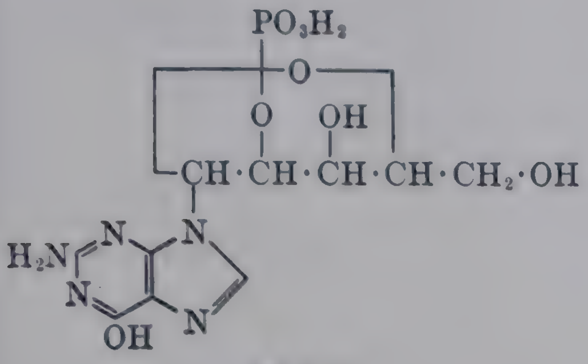
[180] P. A. Levene and W. A. Jacobs, *Ber.* **42,** 2474 (1909).

[181] P. A. Levene and W. A. Jacobs, *Biochem. Z.* **28,** 127 (1910).

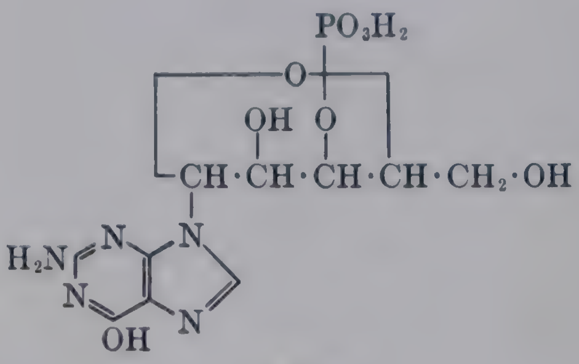
[182] P. A. Levene and A. Dmochowski, *J. Biol. Chem.* **93,** 563 (1931).

[183] M. Knopf, *Z. physiol. Chem.* **92,** 159 (1914).

[184] W. E. Cohn, *J. Am. Chem. Soc.* **72,** 1471 (1950).



XXVIII
Guanosine-2′-phosphate



XXIX
Guanosine-3′-phosphate

h. Cytidylic Acids a *and* b

As the pyrimidine nucleotides are more stable than purine nucleotides towards acids, they may be prepared by hydrolysis of ribonucleic acid with dilute sulfuric acid.[185, 186] By fractional crystallization of the barium salts of the resulting pyrimidine nucleotide mixture, materials known as cytidylic acid and uridylic acid are obtained.[93, 187, 188] Cytidylic acid yields cytidine on neutral hydrolysis.[186] In view of the stability of the pyrimidine nucleotides it is not possible to hydrolyze them to free pyrimidines and ribose phosphates. However, cytidylic acid may be reduced catalytically to dihydrocytidylic acid which, like the corresponding nucleoside dihydrocytosine, is readily hydrolyzed by dilute acids to the dihydropyrimidine.[178] Whereas the phosphate residue in cytidylic acid is rather resistant towards hydrolysis, that in dihydrocytidylic acid has the same order of stability as the phosphate residue in yeast adenylic acid.

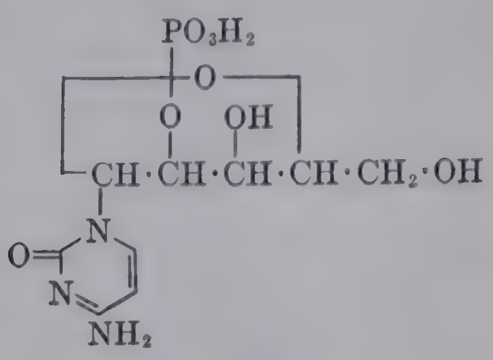
Recently it has been shown that cytidylic acid from ribonucleic acid is a mixture of *a* and *b* isomers.[130, 133] These may be separated by ion-exchange chromatography or by careful fractionation of their salts, and, since they are readily interconvertible in acid solution,[130] do not consume periodate, and are both formed by alkaline hydrolysis of synthetic cytidine-2′,3′-hydrogen phosphate,[173] they must be 2′- and 3′-phosphate (XXX, XXXI). No decision has yet been made as to which is the 2′- and which is the 3′-phosphate on purely chemical grounds. However, careful measurements of physical properties, e.g., spectra, dissociation constants, etc, suggest that cytidytic acid *a* is the 2′-phosphate and cytidylic acid *b* the 3′-phosphate.[189-191]

[185] P. A. Levene, *Biochem. Z.* **17,** 120 (1909).

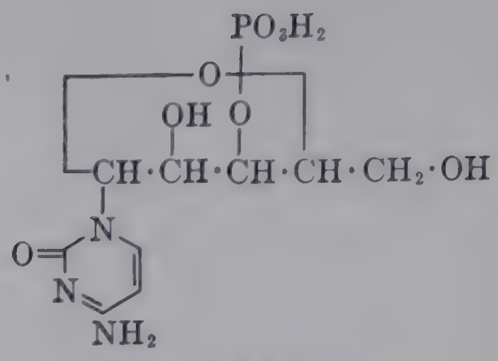
[186] P. A. Levene and W. A. Jacobs, *Ber.* **44,** 1027 (1911).

[187] P. A. Levene, *Proc. Soc. Exptl. Biol. Med.* **15,** 21 (1917).

[188] P. A. Levene, *J. Biol. Chem.* **41,** 1 (1920).



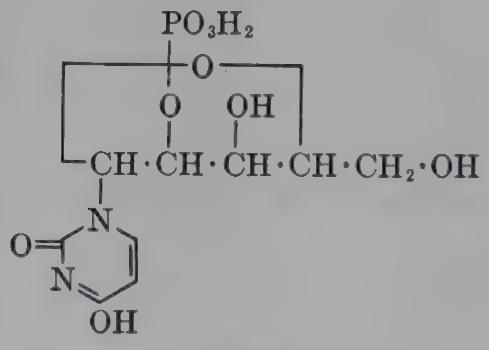
XXX
Cytidine-2′-phosphate



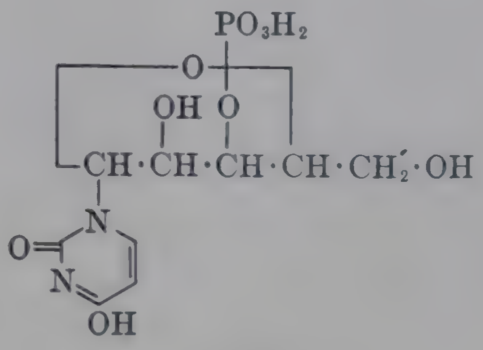
XXXI
Cytidine-3′-phosphate

i. Uridylic Acids a *and* b

The substance known as uridylic acid was isolated, along with cytidy acid, from ribonucleic acid hydrolysates.[186, 188-192] Like cytidylic acid, it c be hydrolyzed to the nucleoside and phosphoric acid. Also, dihydrouridy acid yields uracil, ribose, and phosphoric acid on acid hydrolysis. Uridy acid may be separated into the two isomers, uridylic acid *a* and uridy acid *b*[130] and it has been shown that cytidylic acid *b* is deaminated und alkaline conditions to uridylic acid *b*.[193] Since the conditions of deaminati do not allow phosphate migration, it is concluded that uridylic acid corresponds to cytidylic acid *a* with respect to the position of the phospha group, and uridylic acid *b* corresponds to cytidylic acid *b*. Uridylic acids and *b* are probably the 2′- and 3′-phosphates of uridine, respective (XXXII, XXXIII).



XXXII
Uridine-2′-phosphate



XXXIII
Uridine-3′-phosphate

j. Cyclic Phosphates

Among the products of the action of ribonuclease on ribonucleic acid a pyrimidine nucleotides which differ from any of those described so far.[136,

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[189] L. F. Cavalieri, *J. Am. Chem. Soc.* **74,** 5804 (1952).

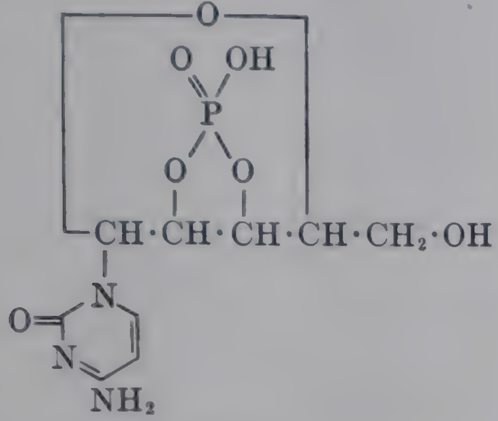
[190] J. J. Fox and L. F. Cavalieri, *Federation Proc.* **12,** 204 (1953).

[191] R. J. C. Harris, S. F. D. Orr, E. M. F. Roe, and J. F. Thomas, *J. Chem. Soc.* **195** 489.

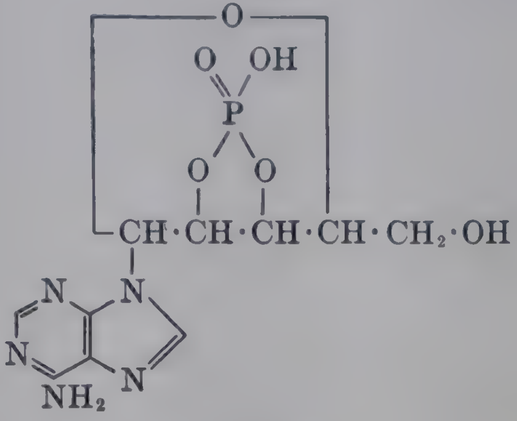
[192] S. J. Thannhauser, *Z. physiol. Chem.* **100,** 121 (1917).

[193] D. M. Brown, C. A. Dekker, and A. R. Todd, *J. Chem. Soc.* **1952,** 2715.

They show the properties of diesters of phosphoric acid (ion-exchange and electrophoresis) and are converted by acids or further action of ribonuclease into mixtures of the *a* and *b* isomers.[173, 193] These properties are consitent with those expected for nucleoside-2′,3′-hydrogen phosphates, and this has been established by direct comparison of the natural substances with synthetic cytidine-2′,3′-hydrogen phosphate (XXXIV) and uridine-2′,3′-hydrogen phosphate.[173]



XXXIV
Cytidine-2′,3′-hydrogen phosphate



XXXV
Adenosine-2′,3′-hydrogen phosphate

Cyclic phosphates of adenosine (XXXV) and guanosine are produced, in addition to the cyclic pyrimidine nucleotides, by very mild alkaline hydrolysis of ribonucleic acid.[136] The cyclic purine nucleotides, although readily hydrolyzed by acids to the *a* and *b* isomers, are not attacked by ribonuclease.[193] The structure of the adenine nucleotide is fully established by comparison with synthetic adenosine-2′,3′-hydrogen phosphate.

k. Deoxyribose Nucleotides

As mentioned before, enzymic or chemical hydrolysis of deoxyribonucleic acid gives pyrimidine deoxyribonucleotides, whereas purine deoxyribonucleotides can only be prepared enzymically. Four monophosphates of the deoxynucleosides have been isolated, namely: thymidylic acid, cytosine deoxyriboside phosphate, adenine deoxyriboside phosphate (XXXVI), and guanine deoxyriboside phosphate.[147-149] In addition a fifth, 5-methylcytosine deoxyriboside phosphate, has been demonstrated in deoxyribonucleic acid hydrolysates by ion-exchange methods.[194] Until recently the phosphate group in the deoxynucleotides was thought to be situated at position 3′ in the sugar chain. In the deoxynucleotides isomerism of the type shown by the ribonucleoside-2′- and -3′-phosphates is not possible and by analogy with these ribonucleotides the 3′-position was thought to be phosphorylated. The isolation of 5′-phosphates from ribonuclease digests of ribonucleic acid invalidates these earlier assumptions. Furthermore, the deoxyribonucleotides are dephosphorylated by a 5′-nucleotidase,[195] and they behave in th manner expected for 5′-phosphates on ion-exchange columns.[196] [Cf. *Cohn* Chapter 6.]

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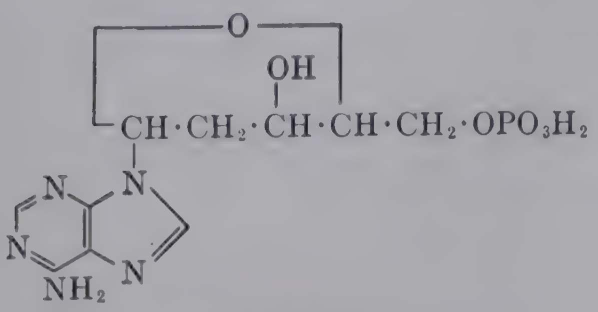
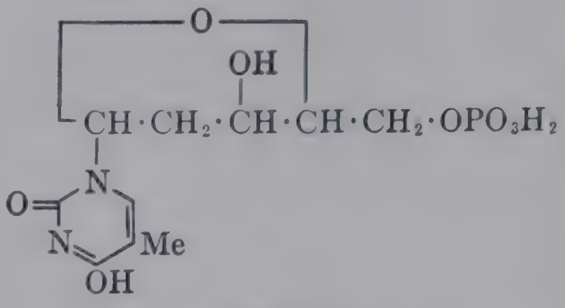
[194] W. E. Cohn, *J. Am. Chem. Soc.* **73,** 1539 (1951).

The thymidine nucleotide has been shown to possess the structure thymidine-5′-phosphate (XXXVII) by synthesis[197] (see p. 175).



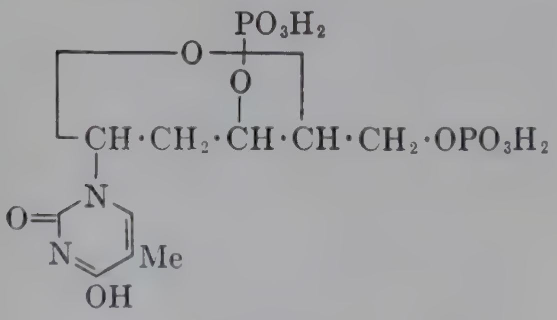
XXXVI
Adenine deoxyriboside-5′-phosphate



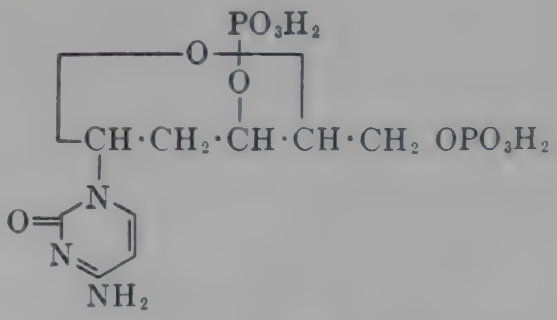
XXXVII
Thymidine-5′-phosphate

l. Deoxynucleoside Diphosphates

Acid hydrolysis of deoxyribonucleic acid gives, in addition to pyrimidine deoxyriboside phosphates, varying amounts of diphosphates of thymidine and cytosine deoxyriboside.[140-145] Chromatographic evidence also suggests the presence in the hydrolysate of traces of the diphosphate of 5-methylcytosine deoxyriboside.[145] Catalytic hydrogenation of thymidine diphosphate, followed by mild acid hydrolysis, gives dihydrothymidine and a reducing sugar diphosphate. These diphosphates are the 3′,5′-diphosphates of thymidine (XXXVIII) and cytosine deoxyriboside (XXXIX) as is shown by their synthesis from thymidine and cytosine deoxyriboside by direct phosphorylation.[145]



XXXVIII
Thymidine-3′,5′-diphosphate



XXXIX
Cytosine deoxyriboside-3′,5′-diphosphate

3. Synthesis of Nucleotides

The formulas assigned to the nucleotides are supported, in some cases, by synthesis. At the present time unambiguous syntheses exist for the nucleoside-5′-phosphates, the cyclic phosphates, and the pyrimidine deoxyribonucleoside diphosphates. Successful methods employ unequivocal protection of appropriate hydroxyl groups on the sugar residue, then phosphorylation and removal of protecting groups.

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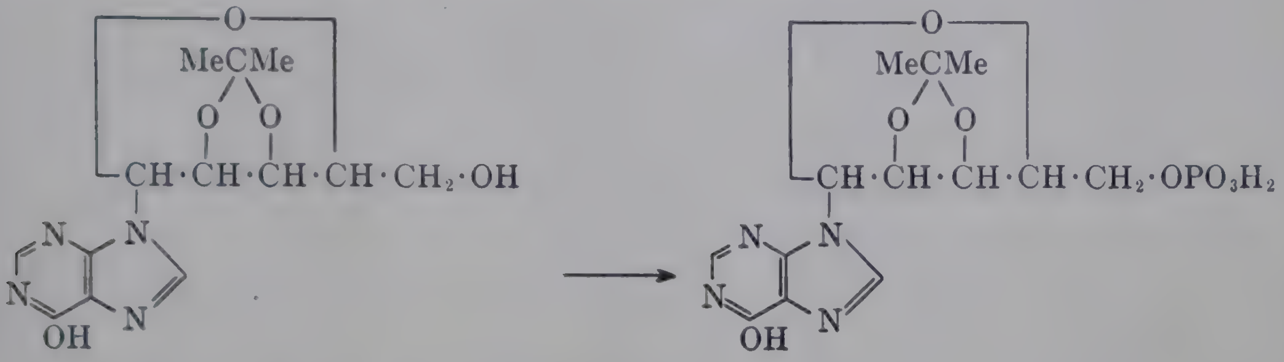
[195] C. E. Carter, *J. Am. Chem. Soc.* **73,** 1537 (1951).

[196] E. Volkin, J. X. Khym, and W. E. Cohn, *J. Am. Chem. Soc.* **73,** 1535 (1951).

[197] A. M. Michelson and A. R. Todd, *J. Chem. Soc.* **1953,** 951.

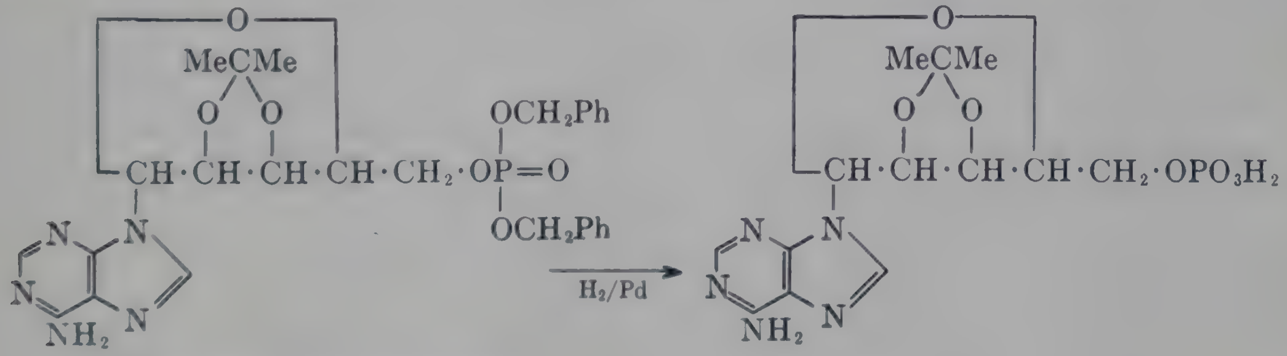
a. The 5′-Phosphates

The most convenient derivatives for the synthesis of ribonucleoside-5′-phosphates are the 2′,3′-isopropylidene compounds. For example inosine-5′-phosphate was first synthesized from 2′,3′-isopropylidene inosine by phosphorylation with phosphoryl chloride in pyridine, then removal of the isopropylidene residue by cautious acid hydrolysis.[17]



A synthesis of adenosine-5′-phosphate from 2′,3′-isopropylidene adenosine follows similar lines.[36] The overall yield in this synthesis is low, however, and the final product difficult to purify. Similarly, phosphorylation of 2′,3′-diacetyladenosine with phosphoryl chloride, then removal of acetyl groups with alkali, gives only low yields of adenosine-5′-phosphate.[38]

The difficulties in these syntheses are probably associated with the nature of the phosphorylating agent. The simultaneous formation of mono-, di-, and triesters and subsequent difficulties arising in the hydrolysis of the phosphorochloridates may be avoided by use of dibenzyl phosphorochloridate.[198] Thus, 2′,3′-isopropylidene adenosine reacts readily with dibenzyl phosphorochloridate in pyridine to give the 5′-dibenzyl phosphate. Benzyl



2′,3′-Isopropylidene adenosine-5′-dibenzyl phosphate groups are removed by catalytic hydrogenation and the isopropyliden residue by acid hydrolysis as before. A good yield of adenosine-5′-phosphat is obtained in this way.[199]

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[198] F. R. Atherton, H. T. Openshaw, and A. R. Todd, *J. Chem. Soc.* **1945**, 382.

Phosphorylation of 2′,3′-isopropylidene cytidine and 2′,3′-isopropyliden uridine with dibenzyl phosphorochloridate, followed by hydrogenation an hydrolysis, gives cytidine-5′-phosphate and uridine-5′-phosphate.[158] Uri dine-5′-phosphate has also been prepared by phosphorylation of 2′,3′-iso propylidene uridine with phosphoryl chloride.[45] Guanosine-5′-phosphat may be prepared from 2′,3′-isopropylidene guanosine and phosphory chloride but not from dibenzyl or diphenyl phosphorochloridate.[158]

b. *The* a *and* b *Isomers*

Syntheses of the 3′-phosphates of adenosine, guanosine, cytidine, an uridine have been reported. These syntheses, however, are either ambiguou or based on erroneous assumptions concerning the structure of intermediates. The ready interconversion of the *a* and *b* series and the unsuspecte difficulty in characterizing the products at the time invalidates much of this work. Direct phosphorylation under various conditions with phosphoryl chloride of adenosine,[200] guanosine, and uridine[201] gives products which were claimed to be identical with the nucleotides obtained from ribonucleic acid. Similarly, phosphorylation of 5′-trityluridine and 5′-tritylcytidine,[39, 202] followed by removal of protecting groups, was thought to give the 3′-phosphates. These syntheses are ambiguous, and it has been shown in the case of adenosine that phosphorylation of the 5′-trityl compound leads to mixtures of *a* and *b* nucleotides.[170]

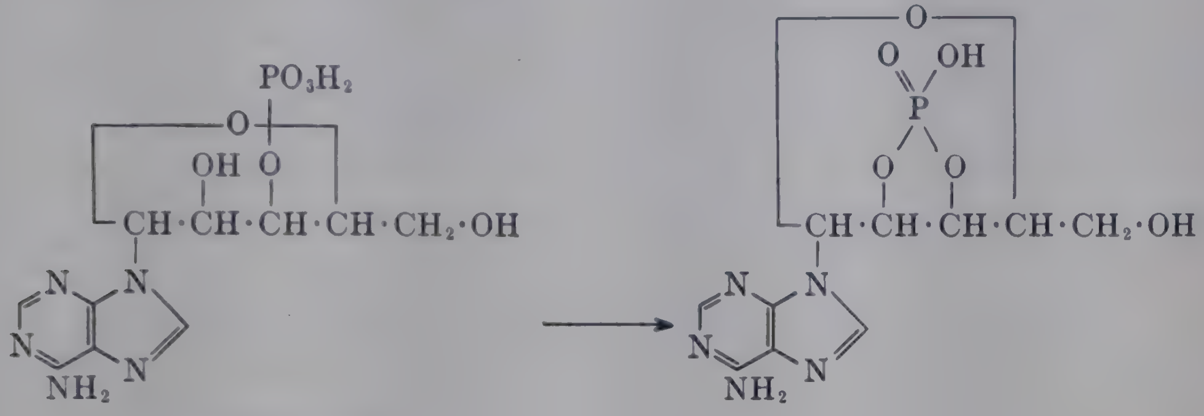
Although unambiguous syntheses of the 2′- and 3′-phosphates of purine and pyrimidine nucleosides have been claimed,[158, 203-205] it is now known that the compounds obtained were 5′-phosphates.[171, 206] These syntheses were based on benzylidene nucleosides, which were believed to be 3′,5′-benzylidene compounds[39, 207] but which have since been shown to be 2′,3′-compounds.

c. *The Cyclic Phosphates*

The four cyclic phosphates obtained by cautious alkaline hydrolysis of ribonucleic acid are prepared from the appropriate *a* or *b* nucleotides by

[199] J. Baddiley and A. R. Todd, *J. Chem. Soc.* **1947,** 648.
[200] G. R. Barker and J. M. Gulland, *J. Chem. Soc.* **1942,** 231.
[201] J. M. Gulland and G. I. Hobday, *J. Chem. Soc.* **1940,** 746.
[202] H. Bredereck, *Z. physiol. Chem.* **224,** 79 (1934).
[203] J. M. Gulland and H. Smith, *J. Chem. Soc.* **1947,** 338.
[204] J. M. Gulland and H. Smith, *J. Chem. Soc.* **1948,** 1527.
[205] J. M. Gulland and H. Smith, *J. Chem. Soc.* **1948,** 1532.
[206] D. M. Brown, L. J. Haynes, and A. R. Todd, *J. Chem. Soc.* **1950,** 408.
[207] J. M. Gulland and W. G. Overend, *J. Chem. Soc.* **1948,** 1380.

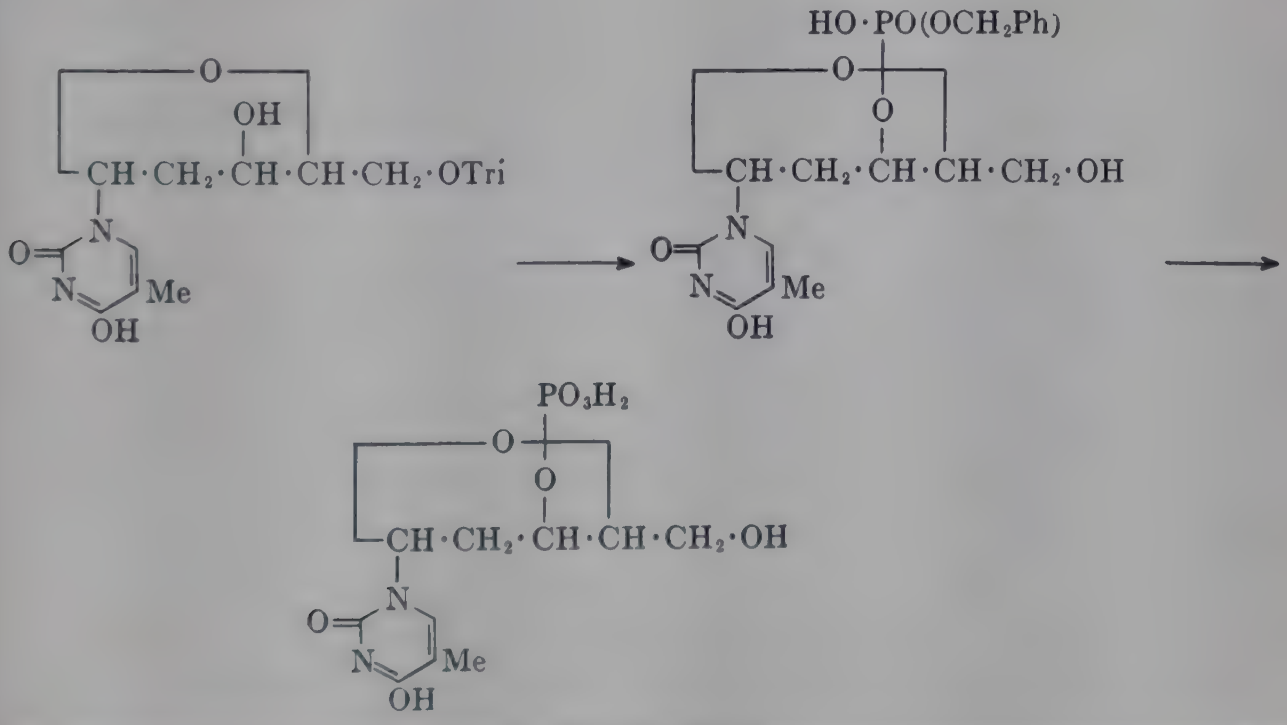
reaction with trifluoroacetic anhydride.[173] Both isomers give the same product, and it is believed that an intermediate mixed anhydride is formed between the phosphate group of the starting nucleotide and a trifluoroacetyl group. The anhydride then effects internal phosphorylation with elimination of the trifluoroacetyl group and production of a cyclic phosphate. A cyclic phosphate of theophylline glucoside has been prepared by direct phosphorylation with phosphoryl chloride in pyridine.[208] Its constitution has not been established.



Adenylic acid *b*

d. Deoxyribose Nucleotides

Thymidine-5′-phosphate and the unnatural 3′-phosphate are synthesized from 5′-tritylthymidine.[197] Direct phosphorylation of the trityl compound with dibenzyl phosphorochloridate followed by treatment with hot acetic acid gives thymidine-3′-benzyl phosphate, from which the benzyl group is removed catalytically.



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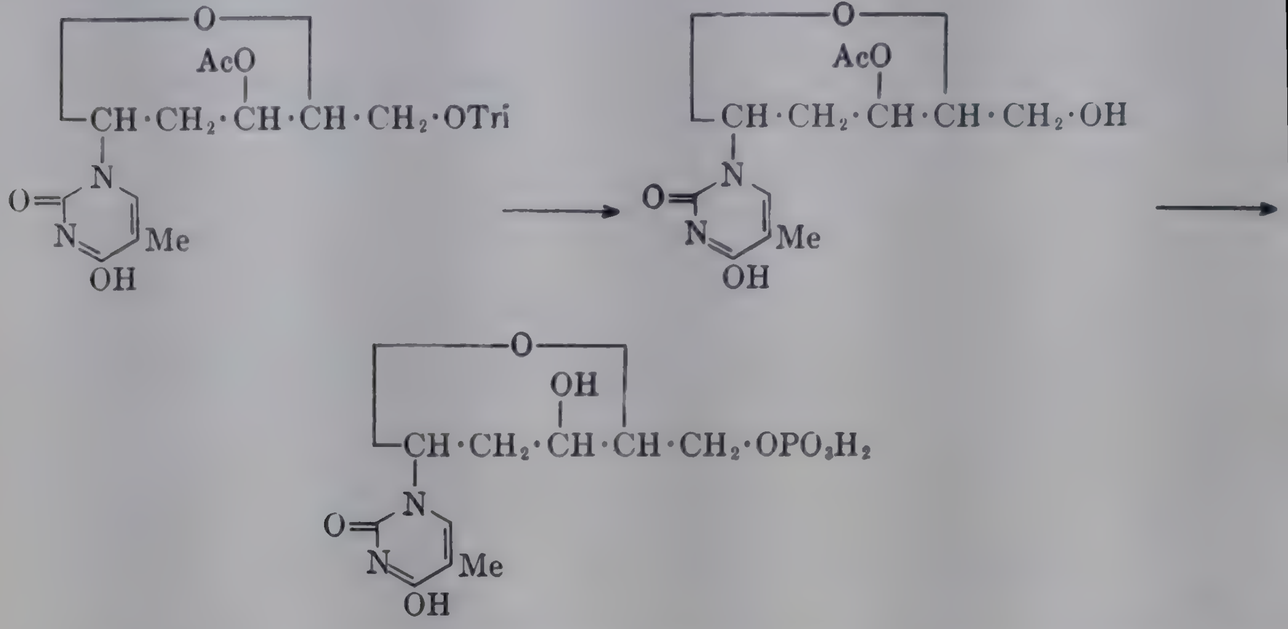
[208] E. Fischer, *Ber.* **47**, 3193 (1914).

Acetylation of 5′-tritylthymidine gives 3′-acetyl-5′-tritylthymidine, which is readily converted into 3′-acetylthymidine by short heating with acetic acid. Phosphorylation of this acetyl derivative with dibenzyl phosphorochloridate, followed by removal of protecting groups, gives thymidine-5′-phosphate.



The 3′,5′-diphosphates of thymidine and cytosine deoxyriboside are prepared by direct phosphorylation of the nucleosides with an excess of dibenzyl phosphorochloridate, then removal of benzyl groups by catalytic hydrogenation.[145]

e. Miscellaneous

Several unnatural nucleotides have been synthesized. Thus adenosine-5′-phenylphosphonate, adenosine-5′-ethylphosphonate, uridine-5′-phenylphosphonate, and uridine-5′-ethylphosphonate are obtained from the appropriate benzyl aryl (or alkyl) phosphonochloridate and the isopropylidene derivatives of adenosine and uridine.[209]

Two diribonucleoside phosphates have been prepared synthetically. The first of these was given the formula diuridine-2′,2″-phosphate, since it was prepared from benzylidene uridine and phenyl phosphorodichloridate.[205] It is now known that the starting material was 2′,3′-benzylidene uridine and not the supposed 3′,5′-compound, consequently it seems probable that the product was diuridine-5′,5″-phosphate. The second member of this group of substances, adenosine-5′ uridine-5′ phosphate has been prepared by an unequivocal route.[210] 2′,3′-Isopropylidene adenosine-5′-dibenzyl phosphate (formula, p. 173) behaves typically as a neutral benzyl ester of phosphoric acid in that it loses one benzyl group on being heated with a

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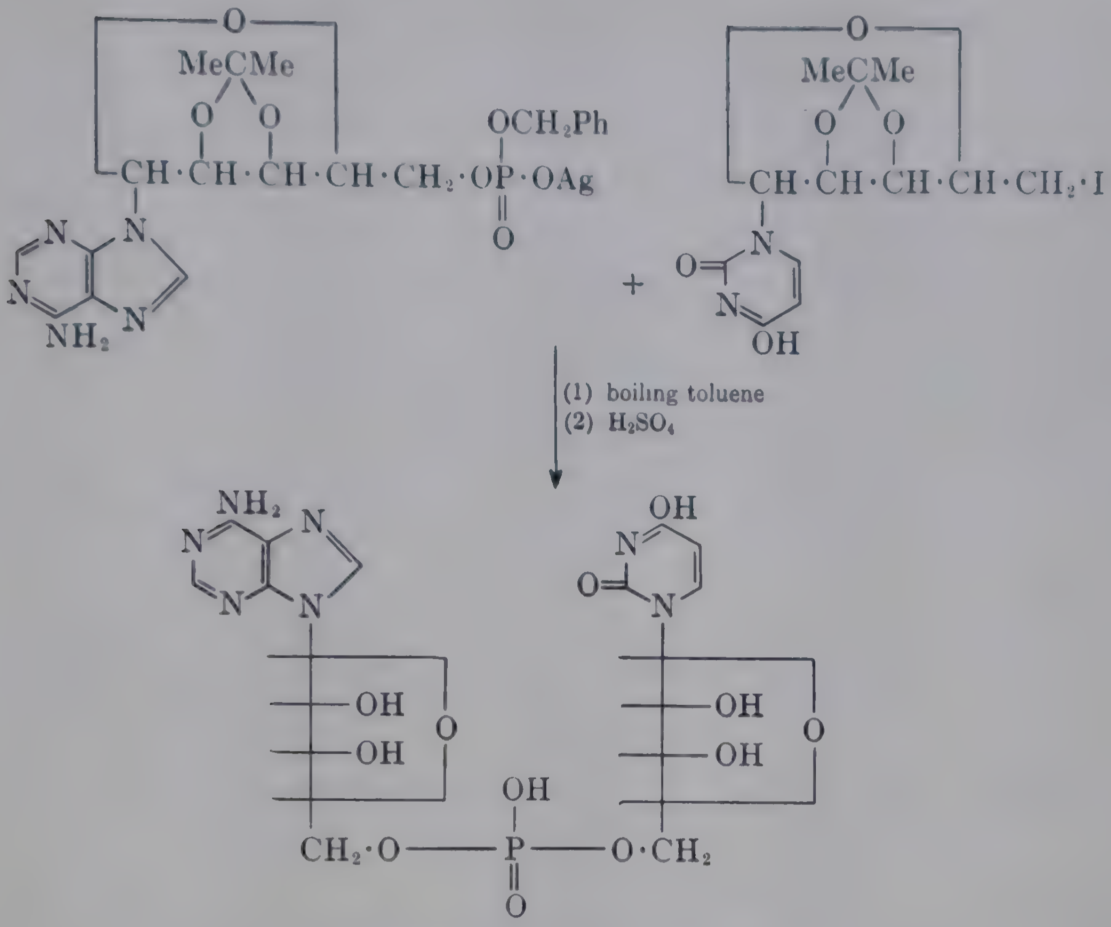
[209] N. Anand and A. R. Todd, *J. Chem. Soc.* **1951,** 1867.

[210] D. T. Elmore and A. R. Todd, *J. Chem. Soc.* **1952,** 3681.



Adenosine-5′ uridine-5′ phosphate

tertiary base.[211] The resulting 2′,3′-isopropylidene adenosine-5′-benzyl phosphate is converted into its silver salt which in turn reacts with 2′,3′-isopropylidene-5′-iodo-5′-deoxyuridine. The isopropylidene and benzyl groups were removed from the resulting neutral ester by gentle acid hydrolysis.

4. Properties of Nucleotides

The nucleotides resemble the nucleosides in many of their physical properties. They are colorless and for the most part crystalline solids with high melting points, frequently decomposing before melting. They are strong acids, soluble in water and insoluble in organic solvents. Their physical properties (Table II) are often rather unreliable for characterization purposes, and this fact was largely responsible for the failure of the early investigators to recognize the multiple nature of some of their materials. The most satisfactory methods for the identication of nucleotides are those employing ion-exchange and paper-chromatographic analysis, often combined with ultraviolet spectroscopy. [Cf. Chapters 6, 7, and 14.]

5. Nucleotide Coenzymes

Nucleotides of adenine and uridine occur as structural units not only of the nucleic acids but also of certain coenzymes. The widespread occurrence

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[211] J. Baddiley, V. M. Clark, J. J. Michalski, and A. R. Todd, *J. Chem. Soc.*, **1949**, 815.

TABLE II

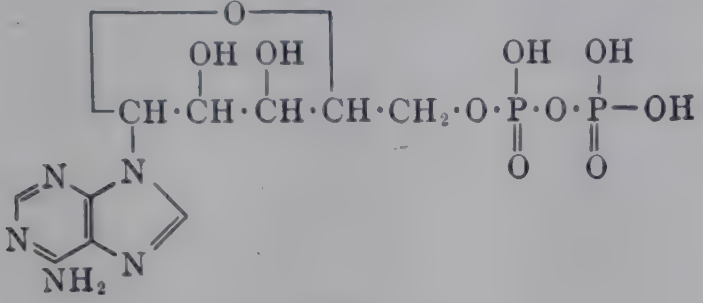
PROPERTIES OF NUCLEOTIDES

Nucleotide	M.p., °C.	$[\alpha]_D$	Derivatives
Inosine-5′-phosphate (muscle inosinic acid)		−18.5° (2.5% HCl)	forms crystalline ammoniu barium, calcium, sodium, an potassium salts
Adenosine-5′-phosphate (muscle adenylic acid)	192	−46.4° (H_2O) −47.5° (NaOH)	acridine salt, m.p. 208°
Guanosine-5′-phosphate	190–200 (dec.)		dibrucine salt, m.p. 210–220 (dec. with prelim. softening
Cytidine-5′-phosphate	233	27.1° (H_2O)	dibrucine salt, softens 185°, m. 215° (dec.)
Uridine-5′-phosphate			barium salt, hexagonal plates dibrucine salt, softens 185 molten at 202°, needles $[\alpha]$ −70.4° (pyridine)
Adenylic acid *a*	187 (dec.)	−84.3° (formamide)	dibrucine salt, m.p. 165–175°
Adenylic acid *b*	197 (dec.)	−58.7° (formamide)	
Cytidylic acid *a*	238–240 (dec.)	20.7° (H_2O)	free nucleotide polymorphous
Cytidylic acid *b*	233–234	49.0° (H_2O)	free nucleotide polymorphous
Uridylic acid *a*			
Uridylic acid *b*			dibrucine salt, $[\alpha]_D$ −58.9° (pyridine)
Thymidine-5′-phosphate		−4.4°	dibrucine salt, rosettes of needles, softening at 140°, m.p. about 175°
Cytosine deoxyriboside-5′-phosphate	183–187	14.4° (Ba salt)	
Thymidine-3′,5′-diphosphate			tetrabrucine salt, softens at 176°, m.p. 182–184°
Cytosine deoxyriboside-3′,5′-diphosphate			tetrabrucine salt, needles sintering at 180°, m.p. 185°

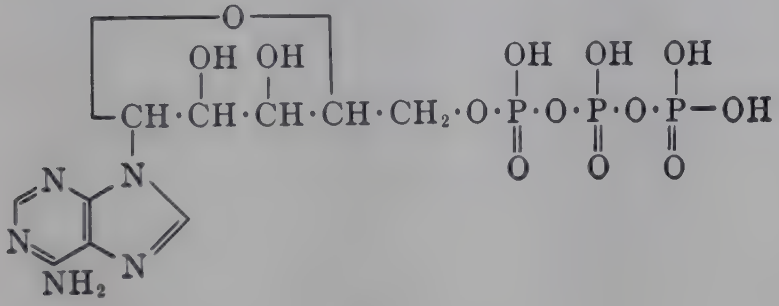
and remarkable versatility of the nucleotide coenzymes warrants their inclusion in this book. Although the chemical transformations which they catalyze are of fundamental importance in all living cells, they are so numerous and intimately interrelated that it is not possible to consider them here and the following sections will be devoted entirely to their chemistry. It will be seen that a common feature of the adenosine and uridine coenzymes is the presence in the molecule of a pyrophosphate linkage.

a. Adenosine Di- and Triphosphate (ADP and ATP)

These two substances participate in the reversible phosphorylation of a very large number of important intermediates in metabolic processes. ATP is usually isolated from muscle[212-214] or after enzymic synthesis from adenosine.[215] Two of its three phosphate groups are removed by acid hydrolysis, the other products being adenine and ribose-5-phosphate. In alkali it gives adenosine-5′-phosphate and pyrophosphoric acid.[216] Titration with alkali indicates the presence of three primary and one secondary phosphate acidic groups, suggesting a linear triphosphate. Positions 2′ and 3′ are unsubstituted since ATP increases the conductivity of boric acid and consumes 1 mol. periodate.[217] These observations indicate the structure XL.



XLI
Adenosine diphosphate



XL
Adenosine triphosphate

ADP (XLI) may be prepared by enzymic dephosphorylation of ATP[218, 219] with the loss of one phosphate group. The presence of a pyrophosphate residue is supported by titration, and the formulas of both these coenzymes have been confirmed by synthesis.

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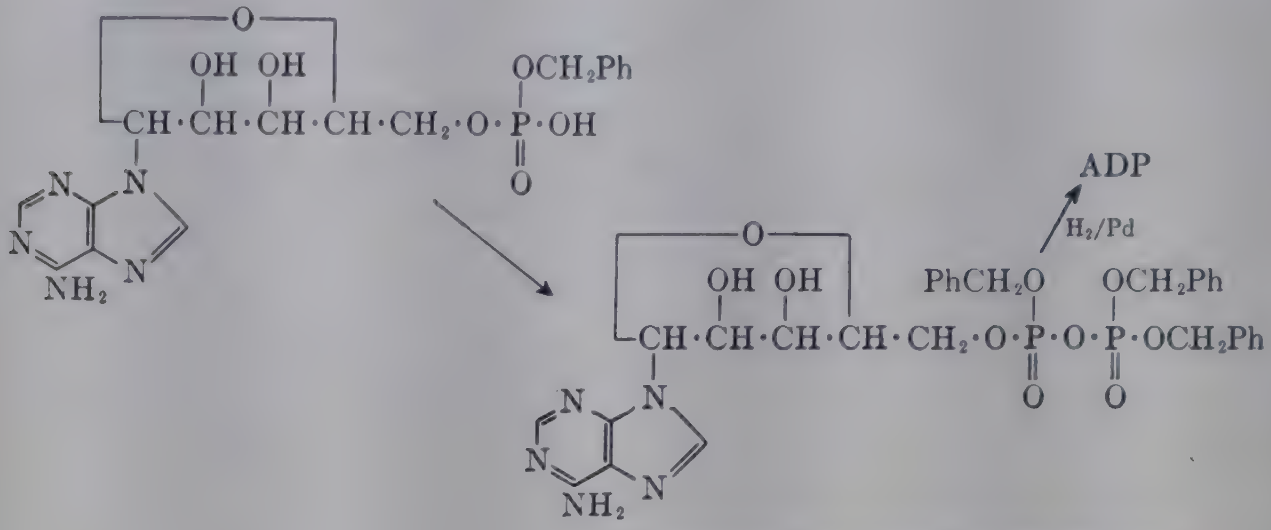
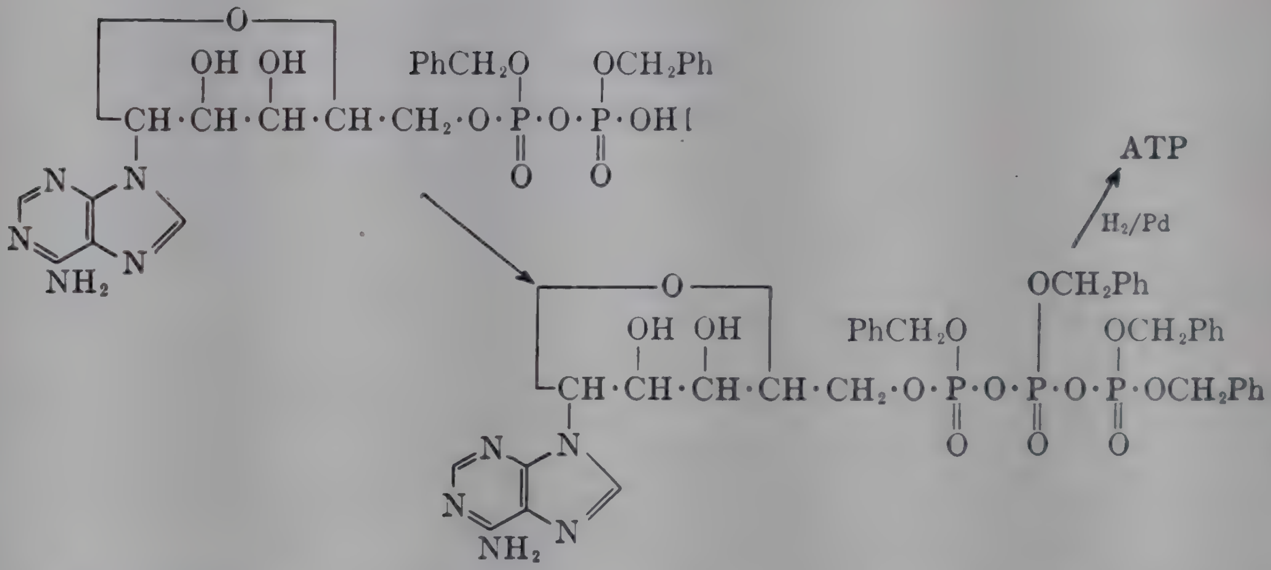
[212] K. Lohmann, *Naturwissenschaften* **17,** 624 (1929).
[213] C. H. Fiske and Y. Subbarow, *Science* **70,** 381 (1929).
[214] G. A. LePage, *Biochem. Preparations* **1,** 5 (1949).
[215] P. Ostern, T. Baranowski, and J. Terszakowec, *Z. physiol. Chem.* **251,** 258 (1938).
[216] K. Lohmann, *Biochem. Z.* **233,** 460 (1931).
[217] B. Lythgoe and A. R. Todd, *Nature* **155,** 695 (1945).
[218] K. Lohmann, *Biochem. Z.* **282,** 104 (1935).
[219] K. Lohmann, *Biochem. Z.* **282,** 120 (1935).

In the synthesis of ADP[199] adenosine-5′-benzyl phosphate is obtained b simultaneous removal in acid of one benzyl and the isopropylidene residu from 2′,3′-isopropylidene adenosine-5′-dibenzyl phosphate (formula, p 173). The silver salt of this monobenzyl ester, with dibenzyl phosphoro chloridate, gives adenosine-5′-tribenzyl pyrophosphate. Catalytic hydrogenation of this gives ADP.



Adenosine-5′-tribenzyl pyrophosphate



When adenosine-5′-tribenzyl pyrophosphate is heated with a teritary base[211] one benzyl group is lost. The resulting dibenzyl ester, as its silver salt, is phosphorylated with a further mol. of dibenzyl phosphorochloridate giving a tetrabenzyl ester of ATP, from which benzyl groups may be removed readily by catalytic hydrogenation.[220] The synthesis has been simplified by direct phosphorylation of the disilver salt of adenosine-5′-phosphate with 2 mol. dibenzyl phosphorochloridate, and then removal of benzyl groups as before. An unstable cyclic intermediate is thought to be formed in this synthesis.[221]

[220] J. Baddiley, A. M. Michelson, and A. R. Todd, *J. Chem. Soc.* **1949**, 582.

[221] A. M. Michelson and A. R. Todd, *J. Chem. Soc.* **1949**, 2487.

b. Di- and Triphosphopyridine Nucleotides (DPN and TPN)

The hydrogen-transporting coenzyme, DPN, was called cozymase when first discovered[222] and is sometimes known as coenzyme I or codehydrogenase I. The closely related TPN[223] differs from it only in possessing an extra phosphate group.[224] On acid hydrolysis DPN gives adenine (1 mol.), nicotinamide (1 mol.), pentose (2 mol.), and phosphoric acid (2 mol.).[225-230] The amino group in the adenine residue of both coenzymes is unsubstituted, since they are deaminated with nitrous acid to the hydroxy analogues without loss of other groups. They are easily reduced to dihydro derivatives which, unlike the coenzymes themselves, are very labile towards acids but relatively stable to alkali. These properties, together with the accompanying changes in ultraviolet spectra, are typical for quaternary pyridinium compounds, and it has been shown that synthetic *N*-glycosides of nicotinamide are very similar in properties to DPN and TPN.[231-233] Furthermore, enzymic hydrolysis of DPN gives *N*-(D-ribofuranosyl)nicotinamide[234-237] (XLII) which, in its dihydro form, is identical with the synthetic glycoside prepared from dihydronicotinamide and acetobromoribofuranose.[238] Only one sugar phosphate (ribose-5-phosphate) is formed by hydrolysis of DPN, and a pyrophosphate linkage is present as shown by titration and the isolation from a hydrolysate of adenosine-5′-pyrophosphate.[239] These facts are consistent with formula XLIII for DPN.[240]

The structures proposed for these coenzymes are strongly supported by enzymic hydrolysis studies.[241, 242] The pyrophosphate linkage of DPN is

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[222] A. Harden and W. J. Young, *Proc. Royal Soc.* (*London*) **B77,** 405 (1906).

[223] O. Warburg and W. Christian, *Biochem. Z.* **254,** 238 (1932).

[224] O. Warburg, W. Christian, and A. Griese, *Biochem. Z.* **282,** 157 (1935).

[225] H. von Euler and K. Myrbäck, *Z. physiol.* **198,** 236 (1931).

[226] H. von Euler and K. Myrbäck, *Z. physiol. Chem.* **203,** 143 (1931).

[227] H. von Euler and K. Myrbäck, *Z. physiol. Chem.* **212,** 7 (1932).

[228] H. von Euler and K. Myrbäck, *Z. physiol. Chem.* **233,** 95 (1935).

[229] H. von Euler, H. Albers, and F. Schlenk, *Z. physiol. Chem.* **237,** 1 (1935).

[230] H. von Euler, H. Albers, and F. Schlenk, *Z. physiol. Chem.* **240,** 113 (1936).

[231] P. Karrer, G. Schwarzenbach, F. Benz, and U. V. Solmssen, *Helv. Chim. Acta* **19,** 811 (1936).

[232] H. von Euler, P. Karrer, and B. Becker, *Helv. Chim. Acta* **19,** 1060 (1936).

[233] P. Karrer, B. H. Ringier, J. Büchi, H. Fritzsche, and U. Solmssen, *Helv. Chim. Acta* **20,** 55 (1937).

[234] F. Schlenk, *Svensk Vet. Akad. Arkiv Kemi* **12B,** 17 (1936).

[235] F. Schlenk, *Svensk Vet Akad. Arkiv Kemi* **14A,** 13 (1941).

[236] F. Schlenk, *Naturwissenschaften* **28,** 46 (1940).

[237] F. Schlenk, *Arch. Biochem.* **3,** 93 (1943).

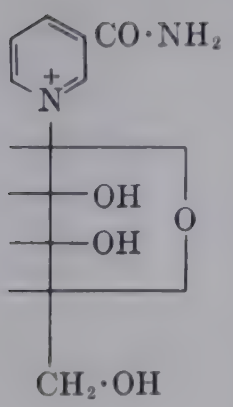
[238] L. J. Haynes and A. R. Todd, *J. Chem. Soc.* **1950,** 303.

[239] R. Vestin, F. Schlenk, and H. von Euler, *Ber.* **70,** 1369 (1937).

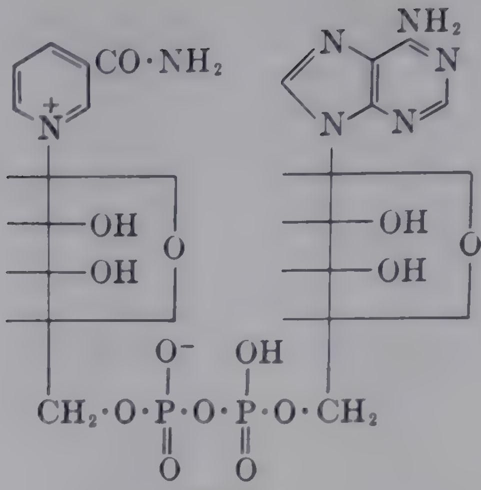
[240] H. von Euler and F. Schlenk, *Z. physiol. Chem.* **246,** 64 (1937).

[241] A. Kornberg, *J. Biol. Chem.* **182,** 779 (1950).

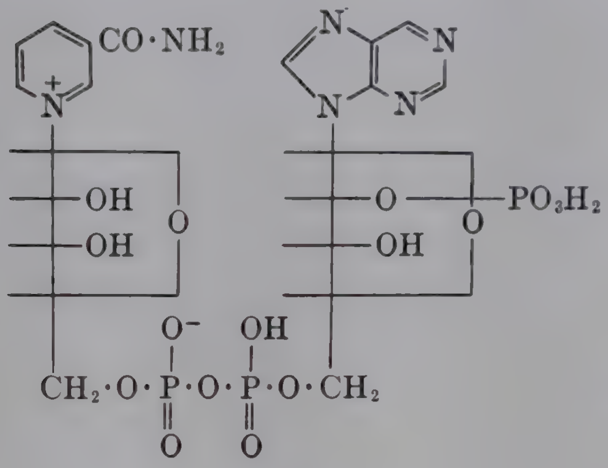
[242] A. Kornberg, *J. Biol. Chem.* **182,** 805 (1950).



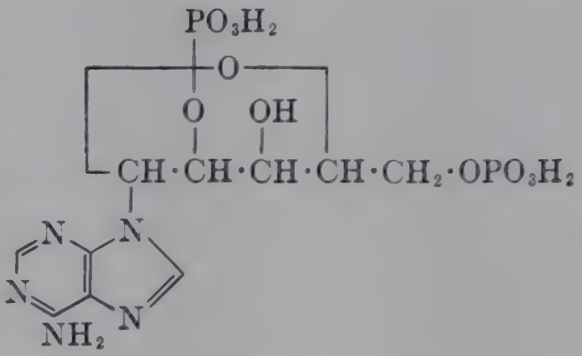
XLII
Nicotinamide riboside



XLIII
DPN



XLIV
TPN



XLV
Adenosine-2′,5′-diphosphate

hydrolyzed by a dinucleotide pyrophosphatase to give nicotinamide nucleoside-5′-phosphate. The coenzyme is resynthesized by this enzyme in the presence of ATP. Also it has been shown that TPN may be synthesized by enzymic phosphorylation of DPN. When TPN (XLIV) is hydrolyzed by the pyrophosphatase a diphosphate of adenosine is obtained. This is adenosine-2′,5′-diphosphate (XLV), since it can be hydrolyzed by a specific 5′-nucleotidase to adenylic acid *a* (adenosine-2′-phosphate).[243]

The coenzyme responsible for the oxidation of cysteinesulfinic acid to cysteic acid is called coenzyme III.[244] Although not yet isolated pure, ultraviolet spectra of the reduced and oxidized forms of the coenzyme suggest that it is a derivative of nicotinamide riboside. Furthermore, it is rapidly destroyed by a pyrophosphatase; thus, it probably contains a pyrophosphate residue. The tentative formula, nicotinamide riboside-5′-pyrophosphate (XLVI) has been assigned to this substance.

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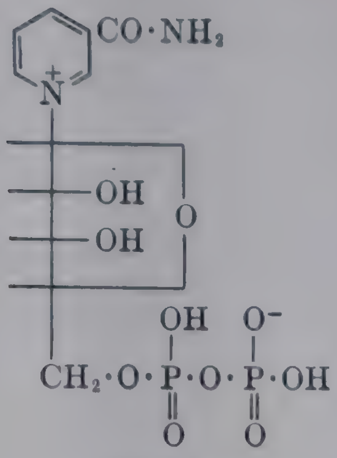
[243] A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.* **186,** 557 (1950).

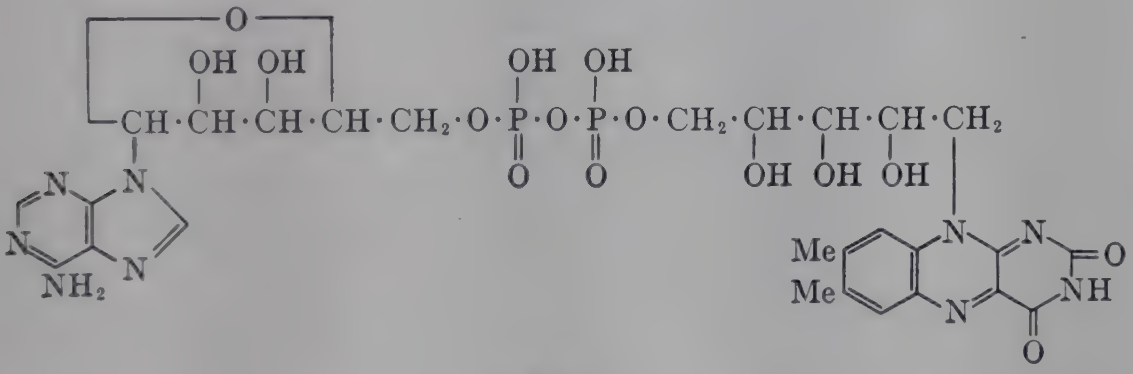
[244] T. P. Singer and E. B. Kearney, *Biochim. et Biophys. Acta* **8,** 700 (1952).



XLVI
Coenzyme III

c. *Flavin Adenine Dinucleotide* (*FAD*)

The coenzyme generally known as flavin adenine dinucleotide is not a true dinucleotide, since one carbohydrate residue is not glycosidically bound. It participates in oxidation-reduction reactions at a higher general level than that for DPN. It is a derivative of riboflavin and gives adenine and phosphoric acid on hydrolysis.[245] An unsubstituted amino group is present since FAD can be deaminated with nitrous acid, and on careful alkaline hydrolysis it gives adenosine-5′-phosphate. In acids it also gives riboflavin-5′-phosphate.[246] A formula for FAD in which the two phosphate residue are joined together as a pyrophosphate (XLVII) is fully confirmed by synthesis.



XLVII
FAD

Riboflavin-5′-phosphate, which has coenzyme activity itself under certain circumstances, has been synthesized by phosphorylation of riboflavin with phosphoryl chloride[247, 248] or from 2′,3′,4′-triacetylriboflavin.[249] FAD has been synthesized in the following manner: A mixed anhydride of dipheny phosphate and benzyl hydrogen phosphite reacts with 2′,3′-isopropyliden adenosine to give the 5′-benzyl phosphite.[250] This is chlorinated with N-chlorosuccinimide to the phosphorochloridate,[251] which then reacts wit the monosilver salt of riboflavin-5′-phosphate. The benzyl group is lost under the experimental conditions employed and the isopropylidene residue removed by acid hydrolysis. From the resulting mixture pure FAD has been isolated.[252]

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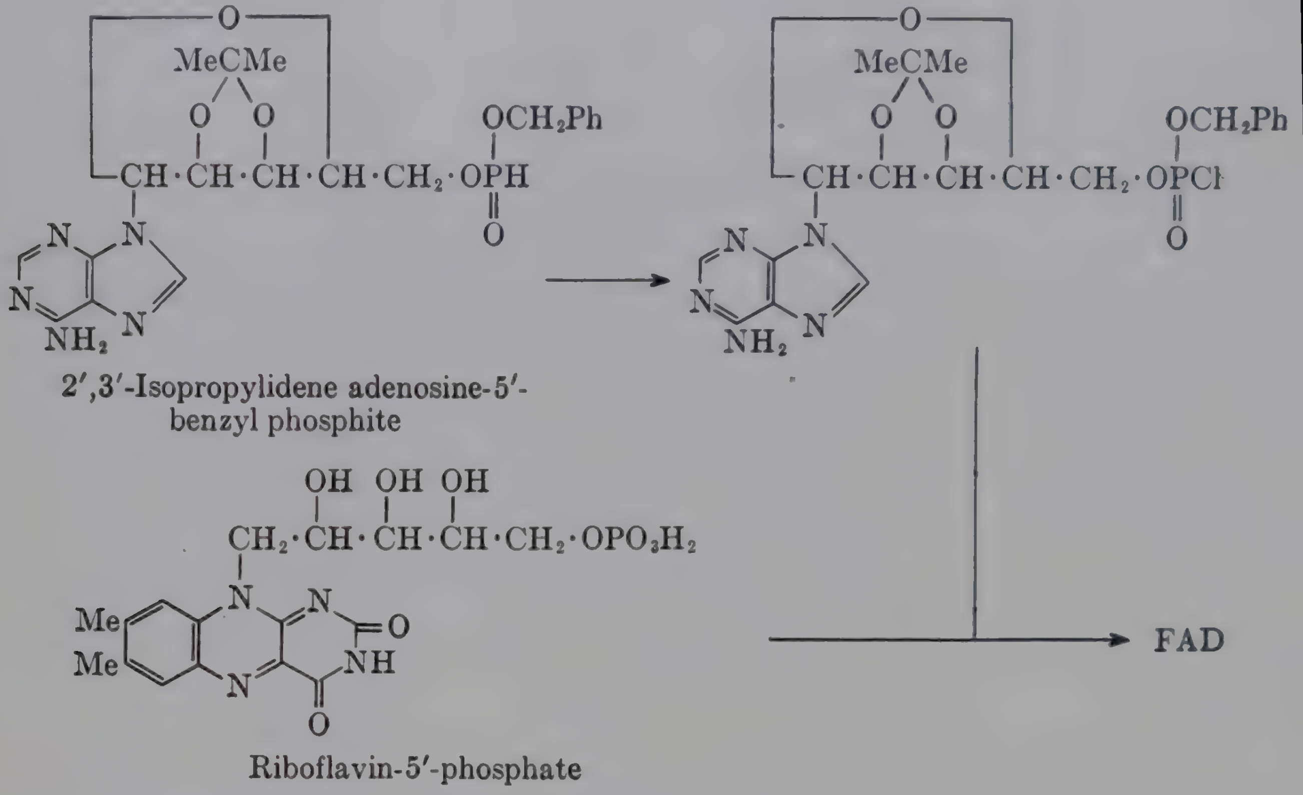
[245] O. Warburg and W. Christian, *Biochem. Z.* **298,** 150 (1938).

[246] E. P. Abraham, *Biochem. J.* **33,** 543 (1939).

[247] R. Kuhn and H. Rudy, *Ber.* **68,** 383 (1935).

[248] H. S. Forrest and A. R. Todd, *J. Chem. Soc.* **1950,** 3295.

[249] R. Kuhn, H. Rudy, and F. Weygand, *Ber.* **69,** 1543 (1936).



d. Coenzyme A (CoA)

The coenzyme responsible for the transfer of acetyl groups, CoA, is a derivative of the vitamin pantothenic acid.[253-255] Chemical or enzymic hydrolysis shows that CoA contains equimolar amounts of pantothenic acid, adenine, pentose, and a sulfur compound[256] subsequently identified as 2-mercaptoethylamine.[257, 258] Three phosphate groups are present.[259] The

[250] N. S. Corby, G. W. Kenner, and A. R. Todd, *J. Chem. Soc.* **1952,** 3669.

[251] G. W. Kenner, A. R. Todd, and F. J. Weymouth, *J. Chem. Soc.* **1952,** 3675.

[252] S. M. H. Christie, G. W. Kenner, and A. R. Todd, *Nature* **170,** 924 (1952).

[253] F. Lipmann, *J. Biol. Chem.* **160,** 173 (1945).

[254] F. Lipmann and N. O. Kaplan, *J. Biol. Chem.* **162,** 743 (1946).

[255] F. Lipmann, N. O. Kaplan, G. D. Novelli, L. C. Tuttle, and B. M. Guirard, *J. Biol. Chem.* **167,** 869 (1947).

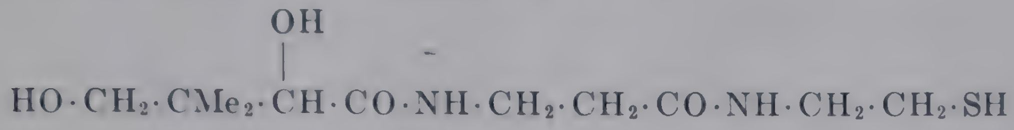
[256] W. H. De Vries, W. M. Govier, J. S. Evans, J. D. Gregory, G. D. Novelli, M. Soodak, and F. Lipmann, *J. Am. Chem. Soc.* **72,** 4838 (1950).

[257] J. Baddiley and E. M. Thain, *J. Chem. Soc.* **1951,** 2253.

[258] J. D. Gregory, G. D. Novelli, and F. Lipmann, *J. Am. Chem. Soc.* **74,** 854 (1952).

[259] G. D. Novelli, J. D. Gregory, R. M. Flynn, and F. J. Schmetz, *Federation Proc.* **10,** 229 (1951).

close relationship between CoA and pantetheine (*Lactobacillus bulgaricus* factor, LBF)[260, 261] is established by the conversion of the former into the latter by enzymic digestion. Pantetheine (XLVIII) may also be converted into CoA enzymically in the presence of ATP.[262]

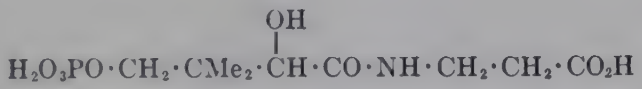


$$\mathrm{HO\cdot CH_2\cdot CMe_2\cdot \overset{\overset{\displaystyle OH}{|}}{C}H\cdot CO\cdot NH\cdot CH_2\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot CH_2\cdot SH}$$

XLVIII

Pantetheine (LBF)

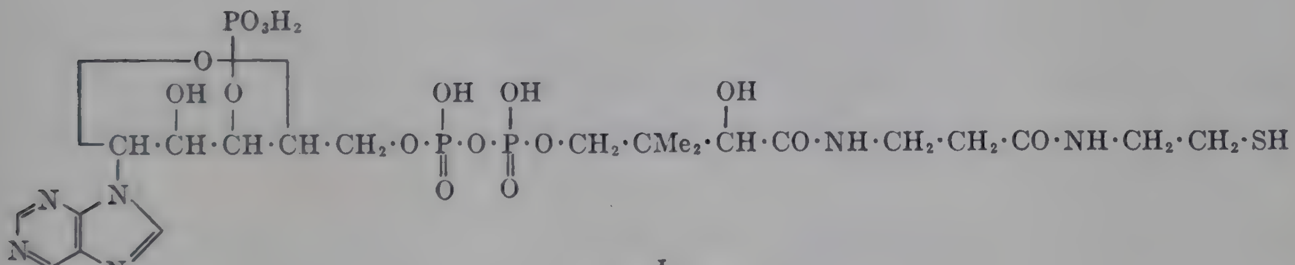
The presence of a pyrophosphate linkage in CoA is indicated by its hydrolysis with a dinucleotide pyrophosphatase[263, 264] and supported by its ready conversion into a cyclic phosphate of pantetheine with dilute alkali.[265, 266] The formation of cyclic phosphates has been observed under similar circumstances with UDPG (see below) and FAD.[248] The pyrophosphate linkage joins the adenosine residue at position 5′ with the pantetheine residue at position 4′. This follows from the observation that acid hydrolysates of CoA contain adenosine-5′-phosphate[257, 259] and pantothenic acid-4′-phosphate (XLIX).[257, 265, 267] The formula for CoA is, then, represented by L.



$$\mathrm{H_2O_3PO\cdot CH_2\cdot CMe_2\cdot \overset{\overset{\displaystyle OH}{|}}{C}H\cdot CO\cdot NH\cdot CH_2\cdot CH_2\cdot CO_2H}$$

XLIX

Pantothenic acid-4′-phosphate



L

Coenzyme A

The third phosphate group is a monophosphate, as shown by enzymic hydrolysis with a monophosphatase, and periodate oxidation[267] indicates that it is situated at positions 2′ or 3′ in the adenosine residue. Since this phosphate is removed by an enzyme which hydrolyzes only *b* nucleotides,

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[260] R. A. McRorie, P. M. Masley, and W. L. Williams, *Arch. Biochem.* **27,** 471, (1950).

[261] G. M. Brown, J. A. Craig, and E. E. Snell, *Arch. Biochem.* **27,** 473 (1950).

[262] R. A. McRorie and W. L. Williams, *J. Bacteriol.* **61,** 737 (1951).

[263] G. D. Novelli, N. O. Kaplan, and F. Lipmann, *Federation Proc.* **9,** 209 (1950).

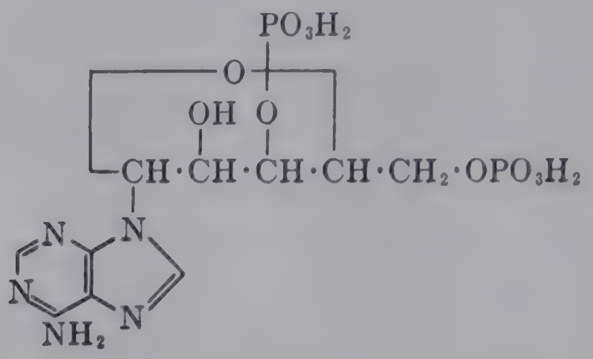
[264] G. D. Novelli, *Phosphorus Metabolism* **1,** 414 (1951).

[265] J. Baddiley and E. M. Thain, *J. Chem. Soc.* **1952,** 3783.

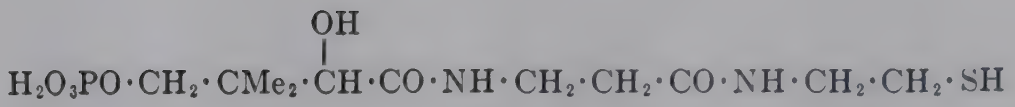
[266] J. Baddiley and E. M. Thain, *J. Chem. Soc.* **1953,** 903.

[267] J. Baddiley and E. M. Thain, *J. Chem. Soc.* **1951,** 3421.

it must be at position 3′ (*b*). This is confirmed by the demonstration that one of the products of pyrophosphatase action on CoA is adenosine-3′,5′-diphosphate (LI), different from the diphosphate obtained from TPN.[268]



LI
Adenosine-3′,5′-diphosphate



$$H_2O_3PO\cdot CH_2\cdot CMe_2\cdot \overset{OH}{\overset{|}{C}}H\cdot CO\cdot NH\cdot CH_2\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot CH_2\cdot SH$$

LII
Pantetheine-4′-phosphate

Strong support for the formula assigned to CoA comes from the synthesis of pantetheine-4′-phosphate (LII) by phosphorylation of pantetheine-$O^{2'}$,*S* dibenzyl ether with dibenzyl phosphorochloridate, then removal of benzyl groups with sodium in liquid ammonia.[269] This compound is identical with the other product of pyrophosphatase action on the coenzyme, and is converted into CoA by liver enzymes in the presence of ATP.[270]

The "trans-acetylation" function of CoA involves the thiol group which, as its thiolacetate, is capable of donating an acetyl group to a variety of substrates.[271] CoA is also involved in the transfer of other important acyl residues in biological systems.

e. *Uridine Diphosphate Glucose* (*UDPG*)

The interconversion of glucose-1-phosphate and galactose-1-phosphate requires an enzyme "galactowaldenase." The coenzyme for this transformation is known as uridine diphosphate glucose (UDPG).[272] It gives on hydrolysis uridine, glucose, and 2 mol. of phosphoric acid. Titration indicates the presence of two primary and no secondary phosphate acidic groups and cautious acid hydrolysis liberates one secondary acidic group, together with glucose. Further hydrolysis liberates 1 mol. of inorganic phosphate.

---

[268] T. P. Wang, L. Schuster, and N. O. Kaplan, *J. Am. Chem. Soc.* **74,** 3204 (1952).

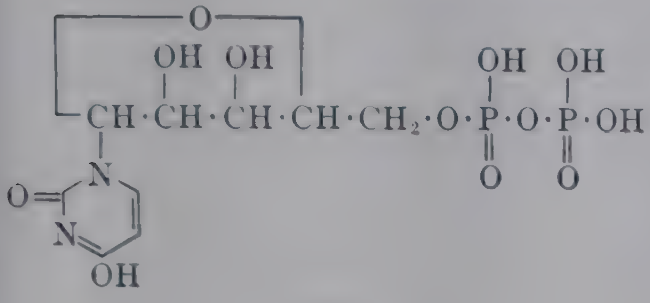
[269] J. Baddiley and E. M. Thain, *J. Chem. Soc.* **1953,** 1610.

[270] J. Baddiley, E. M. Thain, G. D. Novelli, and F. Lipmann, *Nature* **171,** 76 (1953).

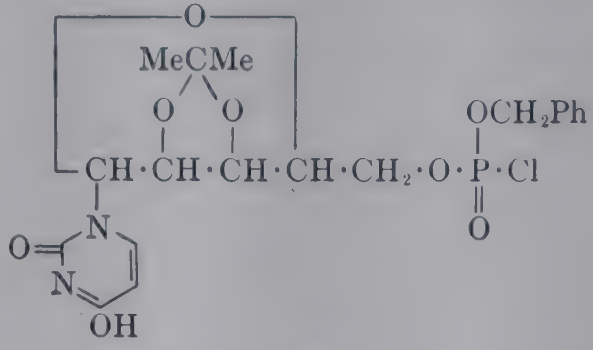
[271] F. Lynen, E. Reichert, and L. Rueff, *Ann.* **574,** 1 (1951).

[272] R. Caputto, L. F. Leloir, C. E. Cardini, and A. C. Paladini, *J. Biol. Chem.* **184,** 333 (1950).

The uridine phosphate formed on hydrolysis consumes 1 mol. of periodate and is identical with synthetic uridine-5′-phosphate.[163] The product of cautious acid hydrolysis is uridine-5′-pyrophosphate (LIII). The pyrophosphate has been synthesized from 2′,3′-isopropylidene-5′-iodo-5′-deoxyuridine (formula, p. 177) by condensing the latter with silver tribenzyl pyrophosphate, then removing protecting groups.[273] It may also be prepared from 2′,3′-isopropylidene uridine-5′-benzyl phosphorochloridate (LIV) and dibenzyl phosphate.[250]

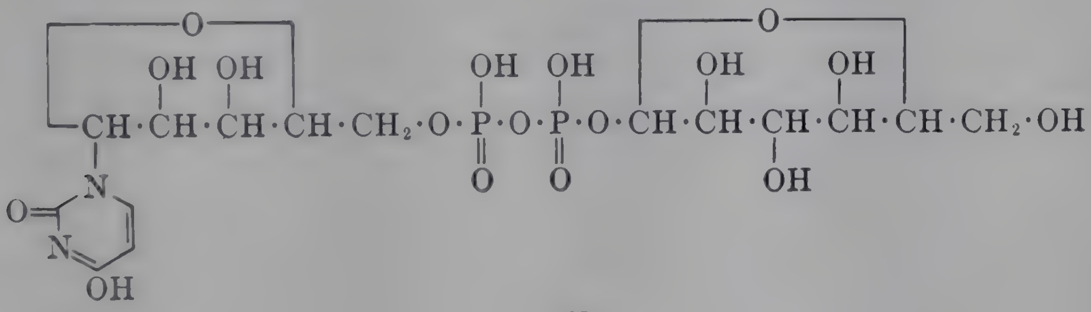


LIII
Uridine-5′-pyrophosphate



LIV
2′,3′-Isopropylidene uridine-5′-benzylphosphorochloridate

The formula LV is established for UDPG:



LV
UDPG

The linkage between the pyrophosphate and glucose residues must be α since cautious alkaline hydrolysis gives glucose-1,2-hydrogen phosphate, which can only be formed from an α-1-phosphate.

Several other uridine diphosphate coenzymes have been encountered in Nature. *Staphylococcus aureus* contains such substances bearing an amino sugar and amino acids in the place of glucose.[274] In addition to the galactose analogue of UDPG there is some evidence for the presence in Nature of UDP derivatives of other sugars.[163, 275, 276]

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[273] N. Anand. V. M. Clark, R. H. Hall, and A. R. Todd, *J. Chem. Soc.* **1952,** 3665.

[274] J. T. Park, *J. Biol. Chem.* **194,** 877, 885, 897 (1952).

[275] J. G. Buchanan, J. A. Bassham, A. A. Benson, D. F. Bradley, M. Calvin, L. L. Daus, M. Goodman, P. M. Hayes, V. H. Lynch, L. T. Norris, and A. T. Wilson, *Phosphorus Metabolism* **2,** 440 (1952).

[276] G. J. Dutton and I. D. E. Storey, *Biochem. J.* **53,** XXXVII (1953).

III. Addendum

Introduction

Bacteriophage nucleic acid yields 5-hydroxymethylcytosine on hydroly sis.[277] Cytosine is not present.

Miscellaneous Nucleosides

"Active methionine" has been synthesized from 5′-deoxy-5′-methy thioadenosine and 2-amino-4-bromobutyric acid, $BrCH_2 \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$.[278] 5′-Deoxy-5′-ethylthioadenosine accumulates in yeas which has been grown in the presence of ethionine.[279]

A synthesis of cordycepose has been reported.[280] Spongothymidine ha been shown to consume 1 mol. of periodate without yielding formic acid The pentose liberated upon hydrolysis is believed to be D-xylose;[281] see however, Ref. 115a.

Vicine has been shown to be a glucoside of 2,4-diamino-5,6-dihydroxy pyrimidine. The D-glucopyranosyl residue is probably involved in glycosidi linkage with the hydroxyl group at position 5.[282]

A syntheis of 3-aminoribose, identical with that from puromycin, ha been reported.[283]

A nucleoside, nebularine, occurs in the mushroom *Agaricus* (*Clitocybe nebularis*.[284] On hydrolysis it yields ribose and purine.[285] Its identity with 9-β-D-ribofuranosylpurine has been shown by two syntheses.[286]

Nucleotides

The structure of the adenylic acids has also been established through an unambiguous synthesis of adenosine-2′-phosphate.[287] Partial acetylation o 5′-acetyladenosine yielded only one diacetyladenosine. This was converted into its monobenzyl phosphite and hence into a diacetyladenosine phosphate. Gentle alkaline hydrolysis removed both acetyl groups, yielding pure adenylic acid *a*, uncontaminated with any *b* isomer. Migration of a phosphate group then cannot have occurred in this synthesis. The diacetyladenosine must have been *b*,5′-diacetyladenosine. It yielded *b*,5′-diacetyl-*a*-tosyladenosine on treatment with *p*-toluenesulfonyl chloride and methanolysis of this gave a methyl tosyl-D-ribofuranoside. As this gave 3,5-dimethylribose after methylation and hydrolysis, it follows that the original tosylated diacetyladenosine was 3′,5′-diacetyl-2′-tosyladenosine, and hence that adenylic acid *a* is adenosine-2′-phosphate.

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[277] G. R. Wyatt and S. S. Cohen, *Biochem. J.* **55,** 774 (1953).

[278] J. Baddiley and G. A. Jamieson, *Chemistry & Industry* **1954,** 375.

[279] F. Schlenk and J. A. Tillotson, *J. Biol. Chem.* **206,** 687 (1954).

[280] R. A. Raphael and C. M. Roxburgh, *Chemistry & Industry* **1953,** 1034.

[281] K. Sato, *J. Biochem.* (*Japan*) **40,** 273 (1953).

[282] A. Bendich and G. C. Clements, *Biochim. et Biophys. Acta* **12,** 462 (1953).

[283] B. R. Baker and R. E. Schaub, *J. Am. Chem. Soc.* **75,** 3864 (1953).

[284] L. Ehrenberg, H. Hedström, N. Löfgren, and B. Takman, *Svensk Kem. Tidskr.* **58,** 269 (1946).

[285] N. Löfgren and B. Lüning, *Acta Chem. Scand.* **7,** 225 (1953).

[286] G. B. Brown and V. S. Weliky, *J. Biol. Chem.* **204,** 1019 (1953).

[287] D. M. Brown, G. D. Fasman, D. I. Magrath, A. R. Todd, W. Cochran, and M. M Woolfson, *Nature* **172,** 1184 (1953).

X-ray crystallographic analysis indicates that adenylic acid *b* is the 3′-phosphate.[287]

Comparison of the infrared spectra of cytidine and deoxycytidine phosphates strongly suggests that cytidylic acid *b* is cytidine-3′-phosphate and hence uridylic acid *b* is uridine-3′-phosphate.[288]

Hydrolysis of ribonucleic acid with a crude snake venom yields, amongst other products, 2′,5′- and 3′,5′-diphosphates of uridine and cytidine.[289]

A diphosphate of 5-hydroxymethylcytosine deoxyriboside has been isolated from bacteriophage nucleic acid.[290]

The synthesis of deoxynucleoside phosphates has now been extended to the preparation of the 3′- and 5′-phosphates of deoxycytidine. The latter compound is identical with the deoxycytidylic acid obtained by enzymic hydrolysis of deoxyribonucleic acid.[288]

Nucleotide Coenzymes

Uridine triphosphate[291] and guanosine triphosphate[292] have been isolated from commercial ATP and ATP from rabbit muscle, respectively. Uridine triphosphate has been synthesized enzymically from ATP and uridine-5′-phosphate.[293] A tetraphosphate of adenosine is also present in some samples of ATP.[294] All the mono-, di-, and triphosphates of adenosine, guanosine, uridine, and cytidine have been detected in tumor extracts.[295, 296] Cytidine-5′-phosphate and related compounds have been isolated from *Lactobacillus arabinosus*.[297]

[288] A. M. Michelson and A. R. Todd, *J. Chem. Soc.* **1954,** 34.

[289] W. E. Cohn and E. Volkin, *J. Biol. Chem.* **203,** 319 (1953).

[290] L. L. Weed and T. A. Courtenay, *J. Biol. Chem.* **206,** 735 (1954).

[291] S. H. Lipton, S. A. Morell, A. Frieden, and R. M. Bock, *J. Am. Chem. Soc.* **75,** 5449 (1953).

[292] R. Bergkvist and A. Deutsch, *Acta Chem. Scand.* **7,** 1307, (1953).

[293] A. Munch-Petersen, H. M. Kalckar, E. Cutolo, and E. E. B. Smith, *Nature* **172,** 1036 (1953).

[294] D. H. Marrian, *Biochim. et Biophys. Acta* **12,** 492 (1953).

[295] H. Schmitz, V. R. Potter, R. Hurlbert, and D. White, *Cancer Research* **14,** 66, (1954).

[296] H. Schmitz, *Biochim. et. Biophys. Acta* **14,** 160 (1954).

[297] J. Baddiley and A. P. Mathias, *Chemistry & Industry* **1954,** 277.

Guanosine-diphosphate-mannose has been isolated from yeast.[298] Th nucleotide yields guanosine-5′-phosphate on hydrolysis, contains two phosphate groups, and gives mannose on gentle hydrolysis. From its titratio and other properties it is believed to be a pyrophosphate analogous t UDPG.

An analogue of UDPG in which the glucose residue is substituted b *N*-acetylglucosamine has been isolated from yeast.[299]

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[298] E. Cabib and L. F. Leloir, *J. Biol. Chem.* **206,** 779, (1954).

[299] E. Cabib, L. F. Leloir, and C. E. Cardini, *J. Biol. Chem.* **203,** 1055, (1953).

CHAPTER 5

Hydrolysis of Nucleic Acids and Procedures for the Direct Estimation of Purine and Pyrimidine Fractions by Absorption Spectrophotometry

HUBERT S. LORING

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I. Hydrolysis of Nucleic Acids

a. General Considerations

The possible hydrolytic products of both pentose and deoxypentose nucleic acids, PNA and DNA, respectively, include purine and pyrimidine bases, pentose and pentose phosphate, nucleosides, nucleotides, and oligonucleotides as well as various degradation products derived from the various substances mentioned. It is characteristic of both types of nucleic acid, however, that the purine riboside or deoxyriboside linkage is unus ally labile to acid hydrolysis, while the pyrimidine riboside or deoxyrib side linkage is relatively resistant. Similarly, phosphate ester grou attached to purine nucleosides in the 2′- or 3′-positions are relatively aci labile in contrast to those attached to pyrimidine nucleosides. The free pu ine bases, adenine and guanine, are readily formed during acid hydrol sis of both types of nucleic acids, whereas the pyrimidine bases remain f the most part as mononucleotides in the case of PNA or as nucleoside d phosphates in the case of DNA. As the purine bases are cleaved, reducin groups from the *N*-riboside or *N*-deoxyriboside linkages are liberated an depending on the conditions used, free ribose or deoxyribose may b formed.

The two types of nucleic acids show, however, a characteristically dif ferent behavior in alkali. [Cf. Chapters 10 to 12.] Pentose nucleic acids ar split to mononucleotides by treatment in 1 N alkali at room temperatur in contrast to DNA which is little affected, as far as precipitability by tri chloroacetic acid is concerned, by such treatment. Such procedures hav served, therefore, for the fractionation and estimation of the relativ amounts of DNA and PNA in tissues.[1, 2] [Cf. *Leslie*, Chapter 16.]

1. Acid Hydrolysis of PNA

a. Liberation of Purine Bases and Ribose

Acid hydrolysis has often been used in estimating the relative amounts o adenine and guanine, reducing sugar, labile phosphate, and pyrimidin nucleotides in PNA. It is of value, in using such procedures to estimate th amounts of the respective components in different PNA's, to assess the de gree to which such procedures lead to a quantitative conversion to th respective products.

The purine bases are liberated from PNA by treatment with alcoholi HCl[3, 4] or by hydrolysis with 0.4 N, 1 N, 2 N, or 6 N HCl or H_2SO_4 a 100–120° for 1–2 hours.[4-8] Both types of procedures have been used for th preparation of adenine and guanine from yeast PNA[3, 9(a)] as well as in an alytical procedures for the estimation of purine and pyrimidine components

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[1] G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.* **161,** 83 (1945).

[2] E. Hammarsten, *Acta Med. Scand. Suppl.* **196,** 634 (1947).

[3] P. A. Levene, *J. Biol. Chem.* **53,** 441 (1922).

[4] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176,** 715 (1948).

[5] R. D. Hotchkiss, *J. Biol. Chem.* **175,** 315 (1948).

[6] S. E. Kerr, K. Seraidarian, and M. Wargon, *J. Biol. Chem.* **181,** 761, 773 (1949).

[7] J. D. Smith and R. Markham, *Biochem. J.* **46,** 509 (1950).

[8] H. S. Loring, J. L. Fairley, and H. L. Seagran, *J. Biol. Chem.* **197,** 823 (1952).

[9] W. Jones, "Nucleic Acids," 2nd ed. Longmans Green & Co., New York, 1920: (a p. 107; (b) pp. 41, 44.

There is some question about the complete liberation of the purine bases in 0.5 *N* HCl at 120° for 2 hours,[5, 10] but, in 1 *N* acid at 100° for 1 hour, adenine and guanine are completely liberated as judged by the recovery of adenine from adenylic acid (95 %) and of guanine from sodium guanylate (100 %) by paper chromatography[4] or by the recovery of adenine from adenylic acid after hydrolysis and precipitation as the silver salt (97 %).[8] In 2 *N* H_2SO_4 at 100° purine nitrogen liberated from pancreas PNA reaches a maximum in about 30 minutes, also indicating the complete liberation of the purine bases from this type of PNA under these conditions.[6] The abovementioned experiments show that the purine bases are formed readily on acid hydrolysis of PNA, without appreciable loss, either by paper chromatography or by silver precipitation and without a significant amount of deamination. Other experiments[11] in which an isotope dilution method was used to estimate the purine concentration have indicated that as much as 7–8 % of both adenine and guanine may be destroyed by 1 *N* HCl at 100°. The reasons for the apparent discrepancy between these results and those mentioned above are not apparent. They probably depend in part on experimental technique and in part on experimental error. Direct evidence that hydrolysis in 1 *N* HCl or H_2SO_4 at 100° for 1 hour liberates the purine bases quantitatively is the recovery of from 98–99 % of the nitrogen of some samples of yeast PNA as known purine and pyrimidine components.[7, 8]

In agreement with the accepted theory that PNAs are polynucleotides and that adenine and guanine are formed from adenylic and guanylic acids, respectively, is the accompanying formation of reducing groups and inorganic phosphate, as adenine and guanine are liberated during acid hydrolysis. The experiments of Levene and co-workers[12, 13] on the preparation of a ribose phosphate from xanthylic acid and the inosinic acid derived from yeast adenylic acid showed that the *N*-riboside linkage could be hydrolyzed at pH 2 before appreciable amounts of inorganic phosphate were formed. [Cf. *Overend and Stacey*, Chapter 2.] In 1 *N* or 2 *N* acid the liberation of purine N likewise is slightly more rapid than the formation of inorganic phosphate.[6] The methods which attempt to relate reducing sugar formation to adenine and guanine liberation involve the conversion of the ribose formed to furfural and its distillation or estimation by colorimetry. [Cf. *Dische*, Chapter 9.] The liberation of inorganic phosphate during acid hydrolysis of PNA has been used directly as a measure of the amount of purine nucleotides present and indirectly as a measure of the acid-resistant pyrimidine nucleotides.[9(b), 14]

[10] M. M. Daly, V. G. Allfrey, and A. E. Mirsky, *J. Gen. Physiol.* **33,** 497 (1950).

[11] R. Abrams, *Arch. Biochem. and Biophys.* **30,** 44 (1951).

[12] P. A. Levene and A. Dmochowski, *J. Biol. Chem.* **93,** 563 (1931).

[13] P. A. Levene and S. A. Harris, *J. Biol. Chem.* **95,** 755 (1932); **98,** 9 (1932); **101,** 419 (1933).

[14] W. Jones, *J. Biol. Chem.* **25,** 87 (1916).

b. Liberation of Inorganic Phosphate and Formation of Pyrimidine Nucleotid

The remarkable stability of the pyrimidine ribonucleotides to mild aci hydrolysis, in contrast to the purine components, was first noted by Leven and Jacobs in experiments in which the mixed nucleotides were prepare as barium salts after a 2-hour hydrolysis of yeast PNA with 2 % H_2SO_4 .[15, 16(a)] Subsequently, after the discovery of nucleotides as products of alkalin PNA hydrolysis, the mixed pyrimidine nucleotides produced by acid hy- drolysis were fractionated into crystalline barium and brucine uridylate and into free cytidylic and uridylic acids.[17, 18] That the phosphoric acid o the pyrimidine components is slowly hydrolyzed by acid was shown by the preparation of cytidine and uridine after acid hydrolysis of the nucleotides[15] and by experiments on the rate of liberation of inorganic phosphate from yeast PNA[9(b), 14] in 5 % H_2SO_4 at 100°. Jones showed that an amount of inorganic phosphate corresponding to 53.9 % of that present in the nucleic acid was liberated during the first 2 hours, but that the amount formed subsequently corresponded to a rate of only 10 mg. of magnesium ammonium phosphate (1.26 mg. P) per gram of nucleic acid per hour. Assuming that the relatively labile phosphate corresponded to that bound to purine nucleosides and correcting the 2-hour period for inorganic phosphate formed from the pyrimidine components, Jones calculated that approximately 50 % (50.7 %) of the nucleic acid phosphate was bound to the purine nucleosides and a similar quantity to the pyrimidine nucleosides in yeast PNA. This occurrence of labile and stable phosphate in nearly equal proportions in yeast PNA has been largely responsible for the tetranucleotide concept of PNA structure.[16(b)] In 2 N H_2SO_4 after 1 hour at 100° Kerr *et al.*[6] have found a slightly higher value for inorganic phosphate formed from yeast PNA, namely about 57–58 %. The rates of phosphate liberation from pure cytidylic and uridylic acids is appreciably higher than the value given by Jones, but the agreement between different investigators is only fair, e.g., approximately 9 % for both nucleotides after 1 hour in 0.1 N H_2SO_4 at 100°[19a, b] as compared with 13.8 % for cytidylic acid under the same conditions or with 15.5 % in 1 N acid.[20] The recovery of purine and pyrimidine components in most of the recently published analyses lies within the range expected from the above mentioned values, viz., 0.50–0.57 and 0.36–0.46 moles per mole of P for purines and pyrimidines, respectively. [Cf. *Magasanik*, Chapter 11.]

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[15] P. A. Levene and W. A. Jacobs, *Ber.* **44,** 1027 (1911).

[16] P. A. Levene and L. W. Bass, "Nucleic Acids," ACS Monograph Series. The Chemical Catalogue Co., New York, 1931: (a) p. 221; (b) p. 274; (c) p. 57; (d) p. 193; (e) p. 265.

[17] P. A. Levene, *Proc. Soc. Exptl. Biol. Med.* **15,** 21 (1917).

[18] S. J. Thannhauser and G. Dorfmüller, *Z. physiol. Chem.* **100,** 121 (1917); **104,** 65 (1919); *Ber.* **51,** 467 (1918).

[19a] A. M. Michelson and A. R. Todd, *J. Chem. Soc.* **1949,** 2476.

[19b] G. R. Barker, J. M. Gulland, H. Smith, and J. F. Thomas, *J. Chem. Soc.* **1949,** 904, find an average value of 7% dephosphorylation for disodium uridylate, $[\alpha]_D^{23} =$ 19.8° (anhydrous) in 0.1 N H_2SO_4 at 100°.

[20] P. A. Levene and E. Jorpes, *J. Biol. Chem.* **81,** 575 (1929).

In estimating the amounts of pyrimidine nucleotides in PNA after acid hydrolysis, it is apparent from the above discussion that appreciable error may result if hydrolysis to nucleosides is neglected.[21, 22] Of significance also is the possible deamination or destruction of the pyrimidine compounds. In 0.4 *N* sulfuric acid at 100° a 2% deamination of cytidine per hour and in 1 *N* HCl a 3–4% deamination of cytidylic acid have been reported.[22, 23]

c. *Liberation of Pyrimidine Bases*

The hydrolysis of the pyrimidine components to the free pyrimidine bases presents considerable difficulty. Procedures for the preparation of cytosine and uracil from yeast PNA involve a 2-hour hydrolysis at 175° in 25% H_2SO_4.[16(c)] Under these conditions there is a considerable deamination of cytosine to uracil, a fact which in the earlier literature placed doubt on the existence of uracil as a nucleic acid component. More recently the liberation of the free pyrimidine bases from PNA by 0.4–6 *N* HCl at 120°,[5] by concentrated formic acid (98–100%) and by 20% HCl at 175°,[4] and by 12 *N* perchloric acid[24] has been studied. The results with 0.4–6 *N* HCl at 120° show an increasing liberation of pyrimidine bases as acid concentration is increased, but the yields of cytosine relative to uracil are low, indicating a considerable degree of deamination under these conditions. In concentrated formic acid cytosine is apparently preserved from deamination to uracil, but the recovery of pyrimidine bases in yeast PNA relative to P amounted to only 0.327 mole per mole, in contrast to values as high as 0.46 found by other methods after 1 *N* acid or alkaline hydrolysis.[7, 8, 25] Hydrolysis of yeast PNA with 12 *N* perchloric acid[24] for 1 hour at 100° causes no appreciable destruction of either adenine, guanine, cytosine, uracil, or thymine, but the recovery of total pyrimidine base when this procedure was applied to yeast PNA, 0.37 moles per mole P, again indicates an incomplete liberation from PNA. At the present time, therefore, under the conditions studied neither 20% HCl, concentrated formic acid, nor 12 *N* perchloric acid can be said to lead to the quantitative liberation of the pyrimidine components of PNA in the form of free bases.

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[21] 21. R. Markham and J. D. Smith, *Biochem. J.* **49,** 401 (1951).

[22] H. S. Loring, J. L. Fairley, H. W. Bortner, and H. L. Seagran, *J. Biol. Chem.* **197,** 809 (1952).

[23] H. S. Loring, and J. Mc T. Ploeser, *J. Biol. Chem.* **178,** 439 (1949).

[24] A. Marshak and H. J. Vogel, *J. Biol. Chem.* **189,** 597 (1951).

[25] E. Chargaff, B. Magasanik, E. Vischer, C. Green, R. Doniger, and D. Elson, *J. Biol. Chem.* **186,** 51 (1950).

d. Formation of Di- and Oligonucleotides

Considerable attention is given in the earlier literature[16(d)] to the occurrence of di- and oligonucleotides as hydrolytic products of nucleic acids. The existence of such compounds, however, was largely disproved when the acid-resistant pyrimidine nucleotide fraction, thought to be a cytidylic uridylic dinucleotide, was successfully separated into cytidylic and uridylic acids. Accordingly the reported isolation by Thannhauser[26] of a trinucleotide after enzymic hydrolysis was never accepted by the Levene school. Recent evidence of the occurrence of products larger than mononucleotides in ribonuclease digests of PNA and deoxyribonuclease digests of DNA is considered in subsequent sections of the book. [Cf. Chapters 6, 8, 10, 11, and 15.]

It has, in addition, been shown[27] that several dinucleotides and one trinucleotide are formed when yeast PNA is treated at room temperature with 15 parts of 6 *N* HCl for 3 minutes at room temperature. The products were successfully fractionated on Dowex 1 (chloride) and were characterized as 5′-[(*b* or *a*)-guanylyl]cytidylic acid *b*, 5′-[(*b* or *a*)-adenylyl]cytidylic acids *a* and *b*, 5′-[(*b* or *a*)-cytidylyl]cytidylic acid *b*, uridylic acid-cytidylic acid dinucleotide, and adenylic acid diguanylic acid trinucleotide.

2. ACID HYDROLYSIS OF DNA

a. Liberation of Purine Bases

The purine bases of DNA are easily removed by mild acid treatment (heating the free nucleic acid in 2% solution at boiling water bath temperature for 10 minutes) apparently without complete degradation of the original polynucleotide structure. The material remaining was early recognized as a complex substance.[16(e)] It was believed free of cytosine as well as adenine and guanine and was named thymic acid[28] because presumably the only base remaining in the original polynucleotide structure was thymine. Feulgen, in a highly important paper,[29] noted that a less degraded product could be obtained under somewhat milder conditions than those mentioned above, namely heating for 40 minutes in slightly acid solution below 80°. This material gave N:P ratios corresponding to approximately equimolar quantities of cytosine and thymine, which were both also isolated after acid hydrolysis. Of special interest to cytology[30] was the demonstration that thymic acid gave a positive fuchsin test which later was correlated with the occurrence of DNA in the cell nucleus. In more recent experiments it was shown that the cytosine-thymine ratio of the original DNA was not altered when DNA was dialyzed at 37° and pH 1.6 and the name "apurinic acid" was proposed for the resulting product.[31] [Cf. *Chargaff*, Chapter 10.]

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[26] S. J. Thannhauser, *Z. physiol. Chem.* **91,** 329 (1914).

[27] R. B. Merrifield and D. W. Woolley, *Federation Proc.* **11,** 258 (1950); *J. Biol. Chem.* **197,** 521 (1952).

[28] A. Kossel and A. Neumann, *Z. physiol. Chem.* **22,** 74 (1896–97).

[29] R. Feulgen, *Z. Physiol. Chem.* **101,** 296 (1918).

[30] J. Brachet, "Chemical Embryology." Interscience Publishers, New York, 1950.

[31] C. Tamm, M. E. Hodes, and E. Chargaff, *J. Biol. Chem.* **195,** 49 (1952).

b. Liberation of Pyrimidine Nucleotides and Free Bases

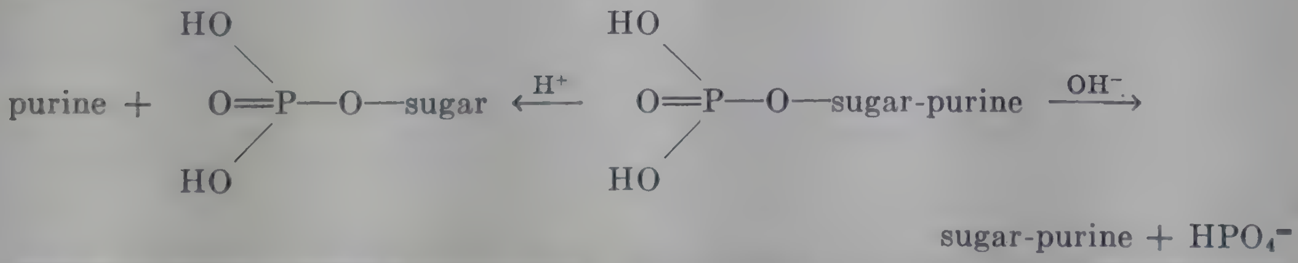
The above-mentioned experiments show the relative ease with which the purine bases are removed from DNA. In quantitative estimations alcoholic HCl (methanol saturated with HCl for 3–5 hours at 50° [4] or 1.5 % methanolic HCl for 20 hours at 37°) [10] has been used. If DNA is heated in 2 % H_2SO_4 for 2 hours under reflux conditions, the remaining polynucleotide structure after removal of the purine bases is further degraded to mixtures of thymine deoxyribodiphosphoric acid and cytosine deoxyribodiphosphoric acid, which may be fractionated and obtained as crystalline compounds. [Cf. *Baddiley*, Chapter 4.] The extent to which other pyrimidine derivatives also may be formed has not been determined.

The hydrolysis of DNA with concentrated formic acid at 175°,[4] with 6 N HCl at 120° [10] or with 12 N perchloric acid[24] leades to a relatively quantitative formation of the free pyrimidine bases. The merits of the different hydrolysis procedures are discussed in Chapters 7 and 10 as regards the recovery of individual bases.

3. Alkaline Hydrolysis of PNA

a. Formation of Nucleosides

The acid instability of the purine ribosidic linkages, in contrast to their alkali stability, was recognized when guanosine (then called vernine) and inosine were discovered.[32, 33] This property of nucleosides has played an important role in establishing the structures of the purine and pyrimidine mononucleotides, since when alkaline hydrolysis is applied to yeast nucleic acid (4–5 % NH_3 for 3.5 hours at about 145°) [34] or to mononucleotides, it is the phosphate ester linkage which is hydrolyzed with the formation of inorganic phosphate and purine and pyrimidine nucleosides. The conversion of inosinic acid to inosine by alkaline hydrolysis or to hypoxanthine and ribose phosphate by acid hydrolysis as carried out by Levene and Jacobs[35] thus laid the groundwork for the structure of all the nucleotides as phosphate esters of nucleosides linked through a hydroxyl group of the sugar as shown below.



$$\text{purine} + (HO)_2P(=O)\text{—O—sugar} \xleftarrow{H^+} (HO)_2P(=O)\text{—O—sugar-purine} \xrightarrow{OH^-} \text{sugar-purine} + HPO_4^{=}$$

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[32] E. Schulze and E. Bosshard, *Z. physiol. Chem.* **10,** 80 (1886).

[33] F. Haiser and F. Wenzel, *Monatsh.* **29,** 157 (1908).

[34] P. A. Levene and W. A. Jacobs, *Ber.* **43,** 3150 (1910).

[35] P. A. Levene and W. A. Jacobs, *Ber.* **41,** 2703 (1908); **42,** 335, (1909); **42,** 1198 (1909); **44,** 746 (1911).

In the original procedure given by Levene and Jacobs[34] for the preparation of guanosine, adenosine, cytidine, and uridine, yeast nucleic acid was heated with concentrated ammonium hydroxide under pressure in an oil bath at 175–180° for 3.5 hours. More recently aqueous pyridine[36] (under reflux for 4½ days), a catalytic hydrolysis with lanthanum,[37] and phosphatase hydrolysis of nucleotides[38-40] have been used to prepare the purine and pyrimidine ribonucleosides in moderate quantities. Their fractionation by starch chromatography[41] in milligram quantities has also been reported.

b. Formation of Nucleotides

As mentioned above, the pyrimidine nucleotides were recognized as acid-resistant phosphate hydrolysis products of yeast PNA in 1911.[16] The discovery that similar organic phosphate compounds could also be produced during alkaline hydrolysis of yeast PNA was made independently by Thannhauser and Dorfmüller[42] and by Jones and Germann[43] when this substance was treated with ammonia under milder conditions (2.3 % NH_3 for 1 hour at 115°) than those used by Levene and Jacobs[34] for the formation of nucleosides. The subsequent purification, isolation, and crystallization of the compounds known as uridylic acid,[17] adenylic acid,[44, 45] cytidylic acid,[18] and guanylic acid[46] resulted from work performed in the three laboratories mentioned on either ammoniacal hydrolysates of yeast PNA or, for the pyrimidine mononucleotides, on acid hydrolysates. The still milder hydrolysis with approximately 1 N NaOH at room temperature was discovered by Steudel and Peiser[47] in experiments in which guanylic and adenylic acids were isolated from yeast PNA after application of this procedure. More recently 1 N alkali at 37°,[1] 0.6 N $Ba(OH)_2$,[48] and 0.1 N NaOH at 100°[49] have been used.

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[36] H. Bredereck, A. Martini, and F. Richter, *Ber.* **74,** 694 (1941).

[37] F. A. Allen and J. E. Bacher, *J. Biol. Chem.* **188,** 59 (1951).

[38] J. M. Gulland and T. F. Macrae, *J. Chem. Soc.* **1933,** 662.

[39] H. S. Loring, M. L. Hammell, L. W. Levy, and H. W. Bortner, *J. Biol. Chem.* **196,** 821 (1952).

[40] P. Reichard, Y. Takenaka, and H. S. Loring, *J. Biol. Chem.* **198,** 599 (1952).

[41] P. Reichard, *Nature* **162,** 662 (1948); *J. Biol. Chem.* **176,** 763 (1949).

[42] S. J. Thannhauser, *Z. physiol. Chem.* **91,** 329 (1914); S. J. Thannhauser and G. Dorfmüller, **95,** 259 (1915); **100,** 121 (1917).

[43] W. Jones and H. C. Germann, *J. Biol. Chem.* **25,** 93 (1916).

[44] W. Jones and R. P. Kennedy, *J. Pharmacol. Exptl. Therap.* **12,** 253 (1918).

[45] S. J. Thannhauser, *Z. phyaiol. Chem.* **107,** 157 (1919).

[46] Guanylic acid was named by Bang in Olaf Hammarsten's laboratory [0. Bang, *Z. physiol. Chem.* **26,** 133 (1898–99)] as a hydrolysis product of pancreas. It was discovered as a component of yeast RNA and crystallized by Levene [P. A. Levene, *J. Biol. Chem.* **40,** 171 (1919); **41,** 483 (1920)].

[47] H. Steudel and E. Peiser, *Z. physiol. Chem.* **114,** 201 (1921); **120,** 292 (1922).

[48] H. S. Loring, P. M. Roll, and J. G. Pierce, *J. Biol. Chem.* **174,** 729 (1948).

c. Liberation of Acid Groups

The earlier experiments mentioned above were concerned primarily with the preparation of the nucleotides in pure form and with their chemistry. It is likely that appreciable amounts of inorganic phosphate, nucleosides, and deaminated products as well as nucleotides were formed under the more severe conditions employed. When a 2% solution of sodium nucleate in 0.1 *N* NaOH is heated at 100°, there is at first a rapid liberation of acid groups[49, 50] followed by a slower liberation of acid until a maximum value is reached corresponding to about 1.08 equivalents per mole of nucleic acid P. As no increase in inorganic phosphate was found under these conditions, it is evident that the acid formed was due to the hydrolysis of phosphate ester linkages in the original nucleic acid structure. Of interest is the fact that the amount of acid formed, 1.08 equivalents per mole of P, is greater by approximately 8% than that expected from a nucleotide polymer containing only diester linkages. The extent to which deamination of purine nucleotides occurs under these conditions has not been determined and is probably negligible. Cytidylic acid is deaminated to the extent of 2 and 12% in 0.01 *N* and 0.1 *N* NaOH, respectively, but is not significantly affected at pH 11.[49] Appreciable deamination, from 10% to 33%, apparently occurs in 1 *N* alkali at 37°.[51]

II. Estimation of Purine and Pyrimidine Components in PNA[22]

1. Chemical Fractionation of Purine Bases and Pyrimidine Nucleotides and Their Estimation by Absorption Spectrophotometry

As discussed above, hydrolysis of PNA with 1 *N* acid at 100° for 1 hour leads to the formation of a mixture of the purine bases and pyrimidine nucleotides with small amounts of pyrimidine nucleosides and possibly oxypurines. As the purine bases form highly insoluble silver salts in acid solution[22, 52, 53] in contrast to either pyrimidine nucleotides or nucleosides, it is possible to effect a relatively quantitative separation of the two types of components by this procedure. If the purine bases are redissolved in dilute HCl and the pyrimidine nucleotide fraction, after removal of silver ions, treated with prostatic phosphatase, two relatively simple binary mixtures

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[49] H. S. Loring, H. W. Bortner, L. W. Levy, and M. L. Hammell, *J. Biol. Chem.* 196, 807 (1952).

[50] H. W. Bortner, Dissertation, submitted to Stanford University for the Degree of Doctor of Philosophy in Chemistry, 1952.

[51] D. H. Marrian, V. L. Spicer, M. E. Balis, and G. B. Brown, *J. Biol. Chem.* **189,** 533 (1951).

[52] R. Feulgen, *Z. physiol. Chem.* **102,** 244 (1918).

[53] S. E. Kerr and K. Seraidarian, *J. Biol. Chem.* **159,** 211 (1945).

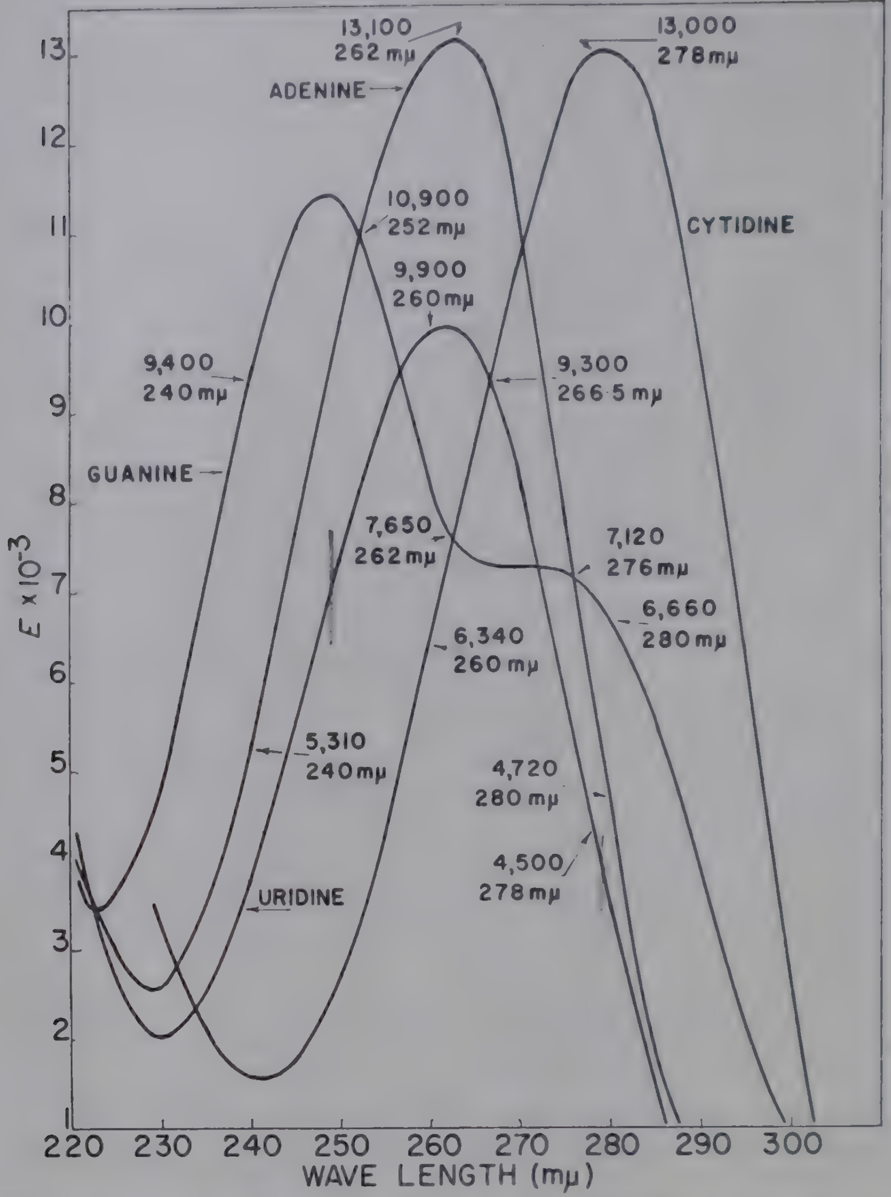

FIG. 1. The ultraviolet absorption spectra of adenine and guanine in 0.1 *N* HCl and of cytidine and uridine in 0.01 *N* HCl.

are obtained of adenine and guanine on the one hand and cytidine and uridine on the other.[54] The ultraviolet absorption spectra of each pair of components in dilute acid as illustrated in Fig. 1[6, 22, 49] are sufficiently different to allow the estimation of the concentration of each component to an extent limited only by the precision of the spectrophotometer and the degree to which other ultraviolet-absorbing impurities may be present in either mixture. [Cf. *Beaven, Holiday, and Johnson*, Chapter 14.]

The analysis of a binary mixture of components, *A* and *B*, having different absorption spectra, is made on the assumption that the total optical density, *D*, at each of two wavelengths, λ_1 and λ_2, is the sum of the densities due to each component at each wavelength.[6, 22, 23] Thus from the Beer-Lambert law two simultaneous equations can be written in which the concentrations of A and B are expressed as a function of their molecular extinction coefficients and the optical densities at the respective wavelengths as follows:[55]

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[54] The relative amounts of the isomeric cytidylic and uridylic acids present in the pyrimidine nucleotide fraction can be estimated directly[6] by absorption spectrophotometry without conversion to nucleosides. The uncertainty in such analyses is larger, however, because the two isomeric cytidylic and uridylic acids present in acid hydrolysates [H. S. Loring, N. G. Luthy, H. W. Bortner, and L. W. Levy, *J. Am. Chem. Soc.* **72,** 2811 (1950); W. E. Cohn, *ibid.* **72,** 2811 (1950)] have somewhat different absorption spectra.[49]

$$C_A E_{A\lambda_1} + C_B E_{B\lambda_1} = D_{\lambda_1}$$

and

$$C_A E_{A\lambda_2} + C_B E_{B\lambda_2} = D_{\lambda_2}$$

C_A and C_B are the respective concentrations of A and B expressed as moles per liter; $E_{A\lambda_1}$, $E_{B\lambda_1}$, $E_{A\lambda_2}$, and $E_{B\lambda_2}$ are the respective molecular extinction coefficients of A and B at λ_1 and λ_2; and D_{λ_1} and D_{λ_2} are the optical densities at the two wavelengths. Solution of the equations for C_A and C_B gives the following:

$$C_A = \frac{E_{B\lambda_1}D_{\lambda_2} - E_{B\lambda_2}D_{\lambda_1}}{E_{A\lambda_2}E_{B\lambda_1} - E_{A\lambda_1}E_{B\lambda_2}}$$

and

$$C_B = \frac{E_{A\lambda_2}D_{\lambda_1} - E_{A\lambda_1}D_{\lambda_2}}{E_{A\lambda_2}E_{B\lambda_1} - E_{A\lambda_1}E_{B\lambda_2}}$$

The pairs of wavelengths selected for the estimation of adenine and guanine or of cytidine and uridine are somewhat arbitrary, but, in order to achieve as high a sensitivity as possible for the detection of either component, it is desirable to use wavelengths where relatively large differences in the molecular extinctions of the two compounds occur, but at which both show appreciable absorption. If one of the wavelength pairs is selected near either end of the ultraviolet spectrum as well, a further check is obtained as to whether or not ultraviolet-absorbing impurities are present which absorb appreciably at the shorter or longer wavelengths. Similarly, by using wavelengths at which intersection points of the respective absorption curves occur, estimates of the total concentration of both components can be made, or, by using a wavelength at which one component fails to absorb, the other can be estimated independently.

In the analysis of PNA performed in the author's laboratory[8, 22] two wavelength pairs, 262 and 280 mμ, and 262 and 240 mμ, were used for estimation of adenine and guanine and both intersection points at 252 mμ and 276 mμ for calculation of total purine bases. The respective equations using molecular extinction values for highly purified samples of adenine and guanine are as follows:

Adenine

$$262\text{–}280\ \mathrm{m}\mu,\ C = \frac{6.66D_{262} - 7.65D_{280}}{5.11 \times 10^4}; \qquad 262\text{–}240\ \mathrm{m}\mu,\ C = \frac{9.40D_{262} - 7.65D_{240}}{8.25 \times 10^4}$$

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[55] If the optical density ratios of the pure substances at the wave lengths chosen are appreciably different, the relative amounts of the two components present can be determined most simply by extrapolation of the optical density ratio.

Guanine

$$262\text{–}280\ \text{m}\mu,\ C = \frac{13.1D_{280} - 4.72D_{262}}{5.11 \times 10^4}; \qquad 262\text{–}240\ \text{m}\mu,\ C = \frac{13.1D_{240} - 5.31D_{262}}{8.25 \times 10^4}$$

Total purine

$$252\ \text{m}\mu,\ C = \frac{D_{252}}{10{,}950}; \qquad 276\ \text{m}\mu,\ C = \frac{D_{276}}{7{,}120}$$

The corresponding equations for cytidine and uridine at 260 and 278 mμ and for total pyrimidine nucleosides at 266.5 mμ are as follows:

$$\text{Cytidine},\ C = \frac{9.90D_{278} - 4.50D_{260}}{1.0 \times 10^5}$$

$$\text{Uridine},\ C = \frac{13.0D_{260} - 6.34D_{278}}{1.0 \times 10^5}$$

$$\text{Total pyrimidine nucleoside},\ C = \frac{D_{266.5}}{9{,}380}$$

2. EFFECT OF VARIOUS TREATMENTS ON THE RECOVERY OF THE PURINE BASES AND THE PYRIMIDINE NUCLEOTIDES

Various recovery experiments have been performed to determine the extent to which known crystalline samples of adenine, guanine, cytidylic acid, and uridylic acid could be recovered after treatment by the various procedures outlined above. The percentage recoveries of mixtures of adenine and guanine after precipitation as silver salts, after heating for 1 hour at 100° in 1 N H_2SO_4 and after a combination of the two procedures are shown in Table I. In the same table the recoveries of cytidylic and uridylic acids as cytidine and uridine, respectively, are shown after dephosphorylation by prostatic phosphatase and after acid hydrolysis and dephosphorylation both without and with the addition and removal of Ag^+. Because the recovery of cytidylic acid is low (96–97%) and the recovery of uridylic acid high (102–104%) after acid hydrolysis, it appears that, as with cytidine under similar conditions,[23] a 3–4% deamination of cytidylic acid occurs after heating a mixture of these substances in 1 N H_2SO_4 at 100° for 1 hour. Other recovery experiments on mixtures of both purine bases and pyrimidine nucleotides gave similar recoveries ranging from 98–101% of the amounts used, provided a correction for 3.5% deamination of cytidylic acid was made. It was furthermore shown that the pyrimidine nucleoside fraction could be filtered through Dowex 1 bicarbonate without loss and that added aromatic amino acids and the traces of purine bases remaining owing to the slight solubility of the silver purines were effectively removed by this treatment.

3. EXPERIMENTAL PROCEDURE FOR THE ANALYSIS OF RIBONUCLEIC ACIDS BY ABSORPTION SPECTROPHOTOMETRY

In analyzing PNA samples by the above-mentioned procedures, it is desirable to relate the purine and pyrimidine composition to their nitrogen and phosphorus contents. In the procedure described below, the Ma and Zuazaga method[56] for nitrogen (micro-Kjeldahl) and a modified Fiske-Subbarow procedure[49] were used.

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[56] T. S. Ma and G. Zuazago, *Ind. Eng. Chem., Anal. Ed.* **14,** 280 (1942).

TABLE I

Per Cent Recovery of Purine Bases and Pyrimidine Nucleotides After Various Treatments

Treatment	Adenine		Guanine		Total purine	
	262–280 mμ	262–240 mμ	262–280 mμ	262–240 mμ	252 mμ	276 mμ
Acid hydrolysis N H_2SO_4, 1 hr., 100°	99	99	101.5	101.5	100.5	100
Precipitation as silver salts	99	98	99	100	98.5	98
Acid hydrolysis and precipitation as silver salts	98	98	99	99	99	98

Treatment	Cytidine		Uridine	Total pyrimidine
	260–278 mμ	295 mμ	260–278 mμ	267 mμ
Dephosphorylation by acid phosphatase	100	102	102	100
Acid hydrolysis and dephosphorylation	97	96	104	100
Acid hydrolysis, Ag^+ added and removed, and dephosphorylation	96	96	102	99
Dephosphorylation and Dowex 1 column (amino acids present)	100	98	99	99

A 150-mg. sample[57] in about 30 ml. of 1 N sulfuric acid in a test tube capped with a small beaker is heated for 1 hour in a boiling water bath. The solution is filtered through a sintered glass funnel to remove a few particles of material presumed to be coagulated protein, and the filtrate and several washings are diluted to 50 ml. in a volumetric flask. Portions are analyzed directly for total nitrogen and, after suitable dilution, for total phosphorus.

For the determination of purine and pyrimidine content, 10-ml. portions of the hydrolysate, in triplicate, are placed in 15-ml. centrifuge tubes. The following procedures are carried out in an identical manner on each of these aliquots as well as on a 10-ml. sample of 1 N H_2SO_4, which provides a reagent blank for all subsequent optical density measurements. The pH of the solution is brought to about 1.0 by the addition of 11 N KOH, and, after warming to 90°, 1 ml. of a 20% $AgNO_3$ solution is added to precipitate the adenine and guanine as silver salts. The suspension, after standing overnight in the refrigerator, is centrifuged in the cold to pack the precipitate of the silver-purines and a small amount of Ag_2SO_4. The precipitate is washed four times in the centrifuge tube with 3-ml. portions of ice-cold 0.1 N H_2SO_4. The original supernatant solution and the washings are fiiltered through a sintered glass funnel to remove stray particles of the flocculent silver-purines and set aside for ana ysis of the pyrimidine components.

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[57] This amount of nucleic acid diluted as described is convenient when micro-Kjeldahl analyses for nitrogen are to be performed. If the sample is analyzed for purine, pyrimidine, and phosphorus only, the amount used can be decreased to about 2 mg. by decreasing the volumes of reagents used proportionally throughout.[8]

The silver-purine precipitate is subjected to four successive treatments with 10-m portions of 0.1 *N* HCl to extract the free purine bases. In each case the suspension silver salts in the acid is heated in a boiling water bath for 5 minutes with occasion stirring and then allowed to cool for 10 minutes. The solids are sedimented by centrifugation, and the supernatant liquid is poured onto the funnel used in the separation of the purine and pyrimidine fractions. The acid solution is allowed to remai in the funnel for several minutes in contact with a few particles of silver-purine retained in the previous step before suction is applied. The combined filtrates from thi procedure, constituting the pure fraction, are diluted with 0.1 *N* HCl to 50 ml. 10-ml. aliquot diluted to 100 ml. with 0.1 *N* HCl is used for optical density measurements at 240, 252, 262, 276, and 280 mμ. The adenine, guanine, and total purine concentrations are calculated from the optical densities as previously described.

The pyrimidine fraction is warmed to about 70°, 3 ml. of 1 *N* HCl added, and th AgCl precipitate allowed to coagulate and removed by filtration. The precipitate i washed three times by suspension in 5-ml. portions of 0.1 *N* HCl. The combined filtrates are diluted to about 45 ml., the pH of the solution adjusted to 4.7 with NaOH (pH meter), and the volume brought to 50 ml. Two 5-ml. aliquots are treated wit 1 ml. of a filtered phosphatase solution containing 1 mg. of enzyme[39] and the resulting solutions are incubated at 38° for 3 hours to bring about dephosphorylation o the pyrimidine nucleotides. One aliquot, diluted to 100 ml. with 0.1 *N* HCl, is use for optical density measurements at 260, 267, 278, and 295 mμ. Cytidine, uridine, an total pyrimidine concentrations were evaluated as previously described and correcte for 3.5% deamination of cytidylic acid. The values obtained in this way may b slightly high if small amounts of ultraviolet-absorbing amino acids were present initially. The latter may be removed as follows: A second aliquot is adjusted t pH 8.3 with NaOH, the solution filtered through a Dowex 1 (bicarbonate) colum (2 cm. x 3 sq.cm.), and the resin washed with about 70 ml. of 2% $NaHCO_3$. The pH o the combined effluent is carefully adjusted to 1.0 with H_2SO_4 and the volume t 100 ml. Optical density measurements are made and the concentrations of cytidine uridine, and total pyrimidine nucleoside calculated as previously described.

4. Application to Purified Yeast Ribonucleic Acids

The results of the application of the above mentioned analytical procedures to different purified samples of yeast PNA are summarized in Table II in comparison with those of several other investigators. [Cf. *Magasanik*, Chapter 11.] They show that from 97 to 99 % of the nitrogen of commercial sodium ribonucleate or of a carefully prepared nucleic acid sample can be accounted for in terms of known purine and pyrimidine components. Similar almost complete recoveries of the nitrogen of other yeast ribonucleic acid samples are reported in several instances by other workers.[7, 24, 25, 58, 59] In relation to phosphate content the results show that purine and pyrimidine bases occur in very nearly equimolar quantities with phosphorus and confirm the general opinion that ribonucleic acids are essentially poly-

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[58] A. Deutsch, R. Zuckerman, and M. S. Dunn, *Ind. Eng. Chem., Anal. Ed.* **24,** 176 (1952).

[59] G. W. Crosbie, R. M. Smellie, and J. N. Davidson, *Biochem. J.* **54,** 287 (1953).

TABLE II

PURINE AND PYRIMIDINE COMPONENTS OF YEAST RIBONUCLEIC ACID FOUND BY VARIOUS INVESTIGATORS

Sample[a]	Method of hydrolysis	Moles per mole P				P accounted for	N accounted for	Reference
		Adenine	Guanine	Cytidine	Uridine			
Commercial N. A.	Acid	0.275	0.334	0.212	0.248	107	98	7
Commercial Preparation 1	Alkaline	0.280	0.290	0.178	0.203	95.1	89.4	25
	Acid	0.288	0.258	0.165	0.195	90.6	86.0	25
Commercial Preparation 2	Alkaline	0.254	0.265	0.199	0.177	89.5	88.5	25
Preparation 3 bakers' yeast	Alkaline	0.262	0.248	0.214	0.203	92.7	93.6	25
	Alkaline	0.255	0.246	0.202	0.232	93.5	92.2	25
	Acid	0.283	0.233	0.211	0.246	97.3	96.8	25
Preparation 4 bakers' yeast	Alkaline	0.242	0.230	0.183	0.235	89.0	88.5	25
	Alkaline	0.234	0.232	0.175	0.241	88.2	87.4	25
Commercial N. A.	Acid	0.25	0.29	0.18	0.19	91	95	24
Sodium nucleate	Acid	0.250	0.269	0.209	0.240	97	99	8
Bakers' yeast	Acid	0.249	0.272	0.212	0.236	97	97	8
Commercial N. A.	Acid	0.258	0.272	0.195	0.228	95	95	8
Commercial N. A.	Alkaline	0.278	0.273	0.206	0.265	102	102	58
Commercial N. A.	Alkaline	0.237	0.272	0.225	0.237	97		59

[a] The original publications should be consulted for complete descriptions of the samples, the methods of purification, and the methods of hydrolysis used. The cytidine values after acid hydrolysis, with the exception of those of reference 8 have not been corrected for deamination. Commercial N. A. = commercial ribonucleic acid. The preparation numbers given are the same as those of the original publication (Chargaff *et al.*[25]).

nucleotides. A comparison of the various samples analyzed by different investigators reveals differences which appear to be significant. The results indicate that considerable variation occurs in the composition of samples of yeast ribonucleic acid, depending on the procedure employed during their preparation and purification.[60] Because of such variation it appears that a better characterization of a particular ribonucleic acid lies in the direct analysis of the purified nucleoprotein or of the particulate component containing the nucleic acid.

5. APPLICATION TO PURIFIED NUCLEOPROTEINS, PARTICULATE COMPONENTS OF CELLS, AND TISSUES

The analysis of the ribonucleic acid occurring in a purified nucleoprotein, in a separated particulate component of cells like mitochondria or microsomes, or in a particular tissue presents a number of difficulties dependi on the amounts of non-nucleic acid purine or pyrimidine or other simil ultraviolet-absorbing compounds that may be present and the variabilit of the respective materials. Of the many types of biological materials avai able for study, the plant viruses approach most closely substances that ca be described as relatively homogeneous, soluble nucleoproteins. Because their high molecular weight, it is relatively simple to separate them by di ferential ultracentrifugation from the many low-molecular-weight com pounds which are associated with living cells and might interfere in th analysis for the purine and pyrimidine components of the ribonucleic acid Analyses have been made on the nucleic acid prepared from purified tobacc mosaic virus[61-63] and many of its strains,[64] by heat or alkali treatment a well as on the trichloroacetic acid–extracted nucleoprotein (TCA nucleo protein)[63] and on the purified virus itself. [Cf. *Magasanik*, Chapter 11.] Th average results expressed as percentage molar proportions of the four bases are summarized in Table III under the respective host species used for the cultivation of the virus. While examination of the combined analyses reveals some differences in the composition of tobacco mosaic virus nucleic acid as reported by the three laboratories, it is also clear that most of the results are in good agreement and the differences within the experimental error. Examination of Table III reveals that quite similar results were found for both the TCA nucleoprotein and its isolated nucleic acid by the spectrophotometric method described above. These results are also in good agreement with those found by Knight for the isolated nucleic acid if the high adenine value reported by him is corrected for a small amount of cytidine likely to be present in the same area of the chromatogram and if a similar correction is made for small losses of unrecovered uridine.[63, 65] The results from the two laboratories are thus in essential agreement for the composition of tobacco mosaic virus nucleic acid when the virus is produced in Turkish tobacco plants. Knight in studies of a number of strains derived from tobacco mosaic virus found a relatively constant composition for the respective nucleic acids, the results suggesting, in fact, that the nucleic acids of different strains of the same virus may have identical compositions.[62, 64]

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[60] For differences in yeast RNA composition depending on the conditions under which yeast is grown see K. Dimroth and L. Jaenicke, *Z. Naturforsch.* **56,** 185 (1950).

The fractionation of broken-cell preparations of tissues into large and small granule fractions as begun by Bensley[67] and modified and improved

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[61] R. Markham and J. D. Smith, *Biochem. J.* **46,** 513 (1950).

[62] C. A. Knight, *J. Biol. Chem.* **197,** 241 (1952).

[63] W. D. Cooper and H. S. Loring, *J. Biol. Chem.*, in press.

[64] F. L. Black and C. A. Knight, *J. Biol. Chem.* **202,** 51 (1953).

[65] R. Markham and J. D. Smith, *Biochem. J.* **49,** 401 (1951).

[66] C. A. Knight, *J. Biol. Chem.* **171,** 297 (1947).

[67] R. R. Bensley, *Biol. Symposia* **10,** 323 (1943).

TABLE III

Percentage Molar Proportions of the Purine and Pyrimidine Bases in the Nucleic Acid from Purified Tobacco Mosaic Virus and from Certain of Its Strains, in the Purified Virus and in the Trichloroacetic Acid-Insoluble Nucleoproetin (TCA Nucleoprotein) as Reported by Different Workers

Strain[a]	Host	Material analyzed	Percentage molar proportions				Reference
			Adenine	Guanine	Cytosine	Uracil	
TMV	White Burley tobacco	Nucleic acid	31.0	29.2	15.5	24.0	61
TMV	Turkish tobacco	Nucleic acid	29.8	25.2	18.5	26.2	62
TMV	Turkish tobacco	Purified virus	28.0	25.8	19.5	26.2	62
TMV	Turkish tobacco	Nucleic acid	28.0	24.0	20.0	28.0	63
TMV	Turkish tobacco	TCA-nucleoprotein	27.8	24.2	19.8	28.6	63
M	Turkish tobacco	Nucleic acid	29.5	26.2	19.3	25.8	62
M	Turkish tobacco	Purified virus	27.5	25.0	20.5	27.5	62
M	Turkish tobacco	TCA-nucleoprotein	27.5	24.5	19.8	28.3	63
Average for six strains produced in Turkish tobacco		Nucleic acid	29.5 ±0.50	25.5 ±0.65	18.5 ±0.55	26.2 ±0.63	62
		Purified virus	28.0 ±0.63	24.7 ±0.65	20.5 ±0.60	26.8 ±0.62	62
Average for TMV and M produced in Turkish tobacco		Nucleic acid TCA-nucleoprotein	27.8 ±0.55	24.0 ±0.63	20.0 ±0.23	28.3 ±0.63	63
Aucuba	Potentate tomato	Nucleic acid	30.0	24.8	19.5	26.2	61
Tomato mosaic	Potentate tomato	Nucleic acid	29.5	26.0	18.3	26.2	61
Y.A.	Turkish tobacco	Nucleic acid	29.8	25.5	18.5	26.5	62
Rib grass	Kawala tobacco	Nucleic acid	29.2	27.0	17.2	26.2	61
	Turkish tobacco	Nucleic acid	29.2	25.8	18.0	27.0	62

[a] The abbreviations are those used by Knight.[66]

TMV = common strain of tobacco mosaic virus

M = Holmes masked strain

Y.A. = Yellow aucuba strain

The six strains include TMV, M, Y.A., $J_{14}D_1$ (a lethal strain in young tobacco plants), GA (a green mottling strain derived from yellow aucuba), and HR (the Holmes Rib-grass strain).

The standard errors of the mean as calculated from the data of Knight[62] and as found by Cooper and Loring.[63] The values are similar to those given by Black and Knight.[64]

TABLE IV

Adenine, Guanine, Cytidine, and Uridine Composition of Mitochondria, Microsomes, and Nonsedimentable PNA Fraction from Normal Swiss Mouse Liver[a]

(μM per 100 mg. TCA and alcohol-ether extracted powder)

Cytoplasmic fractions[b]	Adenine	Guanine	Total purine	Cytidine	Uridine	Total pyrimidine
Mitochondria pool II	2.6	4.9	7.5	3.7	3.4	7.1
Mitochondria pool IV	2.7	5.0	8.0	4.6	3.9	8.2
Mitochondria pool VIa	2.7	4.9	7.7	4.3	2.8	7.0
Mitochondria pool VIb	3.4	5.1	8.7	4.0	2.8	7.0
Microsomes pool II	5.6	10.0	15.7	7.7	3.8	11.6
Microsomes pool III	5.0	9.4	14.4	8.4	5.6	13.9
Microsomes pool IV	4.3	8.2	12.7	6.3	4.7	11.1
Microsomes pool VIa	4.2	8.1	12.4	7.8	5.2	12.6
Microsomes pool VIb	3.9	7.3	11.1	5.9	4.0	10.0
Microsomes pool VIII	4.7	9.5	14.0	7.6	5.3	12.8
Nonsedimentable pool II	1.8	3.6	5.3	3.2	2.1	5.2
Nonsedimentable pool IV	1.4	3.4	4.8	3.1	2.0	5.1
Nonsedimentable pool VIa	1.6	2.8	4.5	3.3	2.4	5.5
Nonsedimentable pool VIb	1.5	3.2	4.6			
Nonsedimentable pool VIII	1.6	3.1	4.6	3.2	2.6	5.6

[a] The means and standard deviations of the means for six separate analyses of a pool of normal mouse liver were as follows: adenine, 2.77 ± 0.05; guanine, 4.98 ± 0.05; total purine, 7.74 ± 0.05; cytidine, 4.44 ± 0.11; uridine, 2.70 ± 0.04; total pyrimidine, 7.13 ± 0.12.

[b] The fresh tissue from each pool was ground in 0.88 *M* buffered sucrose and fractionated by differential centrifugation essentially as given by Hogeboom, Schneider, and Pallade.[69] In VIb the tissue was stored frozen for seven days before grinding.

particularly by Claude[68] and by Hogeboom, Schneider, and Pallade[69] has provided relatively characteristic ribonucleic acid–containing cell components for analytical study. [Cf. *Donace*, Chapter 18, and *Hogeboom and Schneider*, Chapter 21.] Such materials from different animal species and from several types of tissue after extraction with trichloroacetic acid and alcohol-ether to remove acid-soluble and lipid components, respectively, have been analyzed directly for their ribonucleic acid components[70-72] or have been used for the extraction of the ribonucleic acid, which subsequently has been analyzed.[59] Similarly ribonucleic acids prepared from liver and other animal tissues have been extensively analyzed.[25, 59, 72, 73]

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[68] A. Claude, *J. Exptl. Med.* **80,** 19 (1944); **84,** 51 (1946).

[69] G. H. Hogeboom, W. C. Schneider, and G. E. Pallade, *J. Biol. Chem.* **172,** 619 (1948).

[70] A. Marshak, *J. Biol. Chem.* **189,** 607 (1951).

[71] D. Elson and E. Chargaff, *Federation Proc.* **10,** 180 (1951); *Phosphorus Metabolism* **2,** 331 (1952).

[72] F. Leuthardt and B. Exer, *Helv. Chim. Acta* **36,** 500 (1953).

[73] E. Volkin and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1516 (1951).

The results for the extracted ribonucleic acid preparations from several types of tissues and animal species show a wide variation in composition depending on the methods of extraction and analysis that are used.[25, 59, 72, 73] It is also clear that the composition of the ribonucleic acid fractions extracted from isolated mitochondria varies depending on the method used for extraction.[59, 72] Thus, apparently more than one chemical species of ribonucleic acid may be present in this type of cytoplasmic component. The analytical results for the acid and alcohol-ether extracted mitochondria, microsomes, and nonsedimentable ribonucleic acid fractions from mouse liver found by the analytical procedure outlined above are summarized in Table IV. (Unpublished experiments of the author with J. L. Fairley, H. L. Seagran, R. S. Waritz, and M. D. Johnson.) The reproducibility of the method is shown from the values of the standard deviations of the means in a series of six separate analyses of a pool of normal mouse liver powder. Because the variation between pools of mitochondria, microsomes, or nonsedimentable ribonucleic acid fractions is larger than that in a single pool, the results strongly suggest that variations occur in the ribonucleic acid of the different cytoplasmic fractions even in animals under comparable conditions. Under fasting conditions or in mice carrying the Ehrlich ascites tumor variations beyond those normally found also apparently occur.

Of considerable interest in the above-mentioned studies is the relative amount of ribonucleic acid in mitochondria and microsomes as judged by the molar ratios of total purine and purine and pyrimidine base to phosphorus present. If the assumption is made that the base to phosphorus ratio in ribonucleic acid is one, then it can be concluded that significant amounts of trichloroacetic acid–insoluble phosphorus compounds other than nucleic acid are present in such cytoplasmic fractions and to a greater extent in mitochondria than in microsomes. In view of statements in the literature[71, 74] that rat liver mitochondria may contain only small amounts of ribonucleic acid, it may be pointed out that the amounts of purine and pyrimidine bases found in trichloroacetic acid and alcohol-ether extracted mitochondria account for from 60 to 80% of the phosphorus present.

In several instances isolated cell nuclei have also been analyzed for their ribonucleic acid components. The results[70, 71, 75] are apparently highly variable depending on the method of preparation of the nuclei.

[74] V. R. Potter, R. O. Rechnagel, and R. B. Hurlbert, *Federation Proc.* **10**, 646 (1951).

[75] W. M. Mc Indoe and J. N. Davidson, *Brit. J. Cancer* **6**, 200 (1952).

CHAPTER 6

The Separation of Nucleic Acid Derivatives by Chromatography on Ion-Exchange Columns[1]

WALDO E. COHN

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[1] Manuscript prepared under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

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I. Ion Exchange

Ion-Exchange Resins[2]

The basic principles of ion exchange were first observed in clays ar minerals, in particular the zeolites (hydrated aluminum silicates), and le to the use of synthetic zeolites to remove calcium ion from water in exchan for sodium ("water softening"). These synthetic zeolites expanded tl limited range of usefulness of the natural materials, the first analytic application being to remove ammonia from urine for colorimetric determ nation. It remained for the recent rapid developments of resin technolog to produce the synthetic ion-exchange materials which have made possib the spectacular separations of the closely related members of such famili as the rare earths,[3] the amino acids,[4] and the isomeric nucleotides (Sec III.1.*e*). Significant among the properties of these newer materials in th connection are: (1) strength of functional group (strong-acid cation ex changers or strong-base anion exchangers); (2) single functional speci (e.g., nuclear sulfonic acid devoid of phenolic or other acid groups); (3 use of the chemically stable polystyrene resins as the supporting matri (reducing side reactions of the matrix essentially to zero); (4) ability t produce the exchangers in the form of spherical particles (with improve ment in hydrodynamic properties); and (5) control of the degree of cross linking (divinylbenzene) allowing the exchange of substances of high mo lecular weight.

Not all of these advances in technology occurred at once; hence, som of the earlier separations of rare earths and amino acids were performed o aterials of different structure and with less satisfactory results than are urrently expected. The ion-exchange separations of the nucleic acid con- ituents and related products had the advantage of the newer materials om the start. Thus all that has so far been published in this field is omparable, since all utilized the same or very similar materials. For this eason, the discussion of ion-exchange resins which follows will be limited o those few which are currently in use.[5]

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[2] O. Samuelson, "Ion-Exchangers in Analytical Chemistry." John Wiley & Sons New York, 1953.

[3] E. R. Tompkins, J. X. Khym, and W. E. Cohn, *J. Am. Chem. Soc.* **69,** 2769 (1947) B. Ketelle and G. E. Boyd, *ibid.* 2800.

[4] W. H. Stein and S. Moore, *J. Biol. Chem.* **192,** 663 (1951); *et ante.*

2. Ion-Exchange Equilibria[2]

. *Hydrophilic Character*

We will consider only one type of matrix, the polymers of vinylbenzene ross-linked with divinylbenzene to give three-dimensional polystyrene eads of spherical shape. This matrix supports sulfonic acid (strong-acid ation exchanger, such as Dowex-50 or Amberlite-120) or carboxylic acid weak-acid cation exchanger, such as Amberlite-IRC-50) or quaternary mmonium (strong-base anion exchanger, such as Dowex-1, Dowex-2, and mberlite-IRA-400) residues attached to each one of the aromatic rings "nuclear" substitution).[5] The hydrophilic character of these substituents ives the beads, in water, the properties of gels. They swell in water up to imits determined by the degree of cross-linking and show typical Donnan equilibrium effects due to the nondiffusible nature of the highly ionized substituents.

. *The Distribution Coefficient*[2, 6]

The ionized substituents (e.g., resin $SO_3^- H^+$) participate in ionic reactions with diffusible ions (e.g., Na^+) in the surrounding aqueous medium according to the simple exchange reaction

$$(\text{resin } SO_3^-)\ H^+ + Na^+_{\text{soln.}} \underset{2}{\overset{1}{\rightleftarrows}} (\text{resin } SO_3^-)\ Na^+ + H^+_{\text{soln.}}$$

With respect to the diffusible ion ($Na^+_{\text{soln.}}$), sorption consists in favoring reaction 1 (e.g., by low $H^+_{\text{soln.}}$ concentration), desorption or elution in favoring reaction 2 (e.g., high acidity). Under any given set of conditions, an equilibrium can be reached which may be expressed in terms of the mass law but which is usually designated by a "distribution coefficient"

$$K_d(C) = \frac{\text{moles } C \text{ per g. exchanger}}{\text{moles } C \text{ per ml. solution}}$$

---

[5] A complete listing of commercially available ion exchangers, together with their manufacturers, will be found in Samuelson,[2] pp. 262–3. The same volume contains a thorough discussion of all specifications as well as of the principles and applications of ion exchange. See also E. R. Tompkins.[6]

[6] E. R. Tompkins, *Anal. Chem.* **22,** 1352 (1950).

where C is present in small amount compared to the bulk competing ion ions. The distribution coefficient is usually obtained by batch equilib tion, but it is also related directly to the position of the C peak (50 % e tion point) in column chromatography which, in turn, is equal to th point in a breakthrough curve[7] where the concentration of C in the efflue is half of its concentration in the influent $[(C)/(C)_0 = 0.5]$. The ratio of t distribution coefficients of two substances under the same set of conditio is termed the "separation factor,"[2] for it is also the ratio of the distanc from the origin to the peaks of the two substances when eluted under th set of conditions. Thus the distribution coefficients define the peak positio The breadth or sharpness of each peak is related to column length and oth factors (size of particles, cross-linking, rate of flow) which are independe of the distribution coefficient.

c. Nonpolar Affinity

The contrast in character between the polar substituents and the be zenoid matrix underlies many of the anomalies in ion-exchange behavio Whereas the distribution coefficient of a particular solute will depend to large degree upon its charge, it will also depend upon any nonpolar affinit of the solute for the polystyrene matrix and for the ions attached theret The polar attractions are influenced by pH and by complex formatio which affect the sign and degree of charge; the nonpolar attraction is rel tively independent of these factors. Nonpolar affinities exhibit a great temperature dependency than the polar. Reactions involving ions w conform to the principles of stoichiometry; those depending upon nonpola attractions will not conform so exactly and will deviate from equilibr based upon stoichiometric considerations alone.

d. Rate of Reaction

Nonpolar interactions influence the rate of reaction. In general, the rate of reaction between ions where at least one is "strong" (as is the case i the sulfonic and quaternary amine resins) are more rapid and less tem perature-dependent than the nonpolar or "solubility" reactions. The effec of a slow rate of reaction upon the flow rates used in column chromtograph is adverse; a slow rate of reaction will require a slower flow rate to achie the symmetrical bell-shaped elution curve shown by Mayer and Tompkins to depend upon equilibrium conditions. Elevated temperatures can be use to "sharpen" those elution peaks which are diffused due to nonpolar forces as well as to shift their relative positions (see Stein and Moore[4]).

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[7] A breakthrough curve is obtained when the saturation value of a given column fo a particular substance in a given solution is exceeded. When this occurs, the plo of C (concentration in effluent) vs. volume is of a sigmoid nature[2, 6] and approache C_0 (influent concentration) as a maximum.

[8] S. W. Mayer and E. R. Tompkins, *J. Am. Chem. Soc.* **69,** 2866 (1947).

3. Ion-Exchange Chromatography

a. Sorption and Elution

Column chromatography in general requires two steps, the sorption of the sample containing the components to be separated and an elution sequence in which the various components are brought off the column separately. The sorption step usually utilizes conditions of high affinity (high distribution coefficient) between solutes and exchanger (e.g., high charge on the solute, low ionic strength in the solution) to bring about retention of the sample in the topmost layers of the column (Fig. 1). Elution, on the other hand, utilizes conditions in which a larger fraction of the constituent in question is released from the resin (lower distribution coefficient), thus setting up a distribution between solvent and exchanger which permits a reasonable degree of movement of the solute down the column with the solvent flow. The conditions for elution are the opposite of those for sorption; hence, elution can be accomplished by a reduction in charge of the sorbed constituent (by pH adjustment or complex formation or the reverse), or by an increased concentration of competing ion (ionic strength adjustment), or by increased temperature (decreased nonpolar attraction), or by some combination of these.

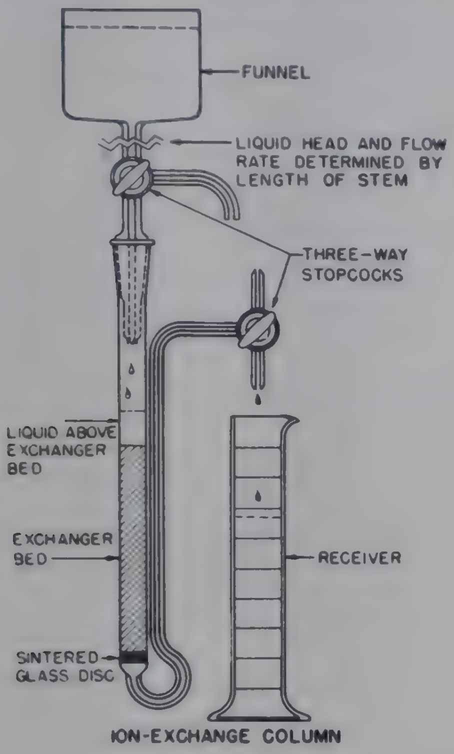

FIG. 1. Ion-exchange column and receiver (after Tompkins).[3, 9]

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[9] E. R. Tompkins, J. Chem. Educ. **26,** 32, 92 (1949).

b. Types of Chromatography[2]

Two methods of exploiting these factors are recognized. In “displac ment chromatography,”[10] the chemical form of the exchanger is chang during the elution sequence (e.g., from a hydrogen-form cation exchang to an ammonium-form, or from a hydroxide-form anion exchanger to chloride-form), and the various components trail the progressive change i form by greater or lesser distances. This method has not had as wid spread practical success as the method of “development chromatography, also called “elution analysis,” in which the salt form of the column remai unchanged from sorption sequence to elution sequence. In the latt method, the influent contains, as its competing, replacing, or bulk ion, th diffusible ion of the exchanger itself. This method has been essentially th only one applied to the separation of nucleic acid constituents. An innovation, in which the eluting sequence is carried out by a gradually changin concentration of eluting agent, instead of changing the solvent discontinuously, has been termed “gradient development chromatography.”[10, 11]

c. Scaling Up

It should be noted that a column separation is also a preparation. The magnitude of the preparation is determined only by the cross-sectional area of the column. Thus analytical separations can be scaled up by proportional increase in the area of the column to prepare larger amounts of material with no loss in resolution.[9] Such large-scale ion-exchange chromatography has been the main source of most of the new nucleotides discovered by analytical ion-exchange chromatography.

The factors and principles just discussed were known in 1948 when the first serious efforts were made to develop precise ion-exchange chromatographic separations of nucleic acid derivatives. Although the attempt was made to find conditions giving good separations with elution curves conforming to those derived from equilibrium considerations, no systematic examination of the many possible separation conditions has as yet been made. Indeed, the stimulus for most of the developments arose from the desire to recover degradation products as an approach to establishing structural relationships in the parent nucleic acid. The separations presented, therefore, do not necessarily indicate the best possible applications of the methods.

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[10] E. Glueckauf, *Nature* **170,** 150 (1952) (as reported in Principles and Applications of Ion Exchange).

[11] H. Busch, R. B. Hurlbert, and V. R. Potter, *J. Biol. Chem.* **196,** 717 (1952).

TABLE I

pK Values of Bases, Ribosides, and Nucleotides[a]

	Bases		Nucleosides[b]		Nucleotides[b]			
	Cationic	Anionic (OH)	Cationic	Anionic (OH)	Cationic	Phosphates		OH
						First	Second	
Purines								
Adenine	$4.1^{c,d,e}$, 4.22^{f}	$9.8^{e,d}$	$3.5^{c,f}$, 3.6^{g}	13^{h}	3.7, 4.4^{i}	0.89	6.01, 6.4^{i}	*j*
Guanine	3.2^{c}	9.6,^{c} 9.36^{k}	1.6, 2.2^{c}	9.16, 9.5^{c}	2.3	0.7	5.92, 6.4^{i}	9.36, 9.7^{i}
Hypoxanthine	$(\sim 2)^{c}$,1.98^{f}	8.7^{g}, $8.9^{c,f}$	1.2^{c}, 1.5^{f}	$8.7–8.8^{c,f,g}$	*j*	1.54	6.04	8.88
Xanthine	$(\sim 0.8)^{c}$	$7.7^{c,g}$ 12^{c}	*l*, $<2.5^{d}$	$5.7^{c,f}$, 6.0^{g}	*j*	—	—	*j*
Pyrimidines								
Uracil	$(\sim 0.5)^{c}$	9.45, 9.5^{m}	*l*	9.17, $9.2^{c,m}$	—	1.02	5.88	9.43
Thymine	$(\sim 0)^{c}$	9.94, 9.9^{m}	*l*	9.8^{m}	—	1.6^{i}	6.5^{i}	10.0^{i}
Cytosine	4.6, $4.5^{c,m}$	12.16, 12.2^{m}	4.22, $4.1^{c,m}$	12.3^{b}, $\sim 13^{c}$	4.2^{c}, 4.6^{i}	0.80	5.97, 6.6^{i}	13.2^{h}
5-Methylcytosine	4.8,^{c} 4.6^{m}	12.4^{m}	*l*	—	4.4^{c}	—	—	—

[a] From Levene and Bass[12] unless otherwise noted.

[b] Ribosides and ribonucleotides except for thymidine and thymidine-5′-phosphate. For isomeric nucleotides, see Table II.

[c] From spectrophotometric observations.[13]

[d] Alberty *et al.* (at 25°C.).[14]

[e] Taylor.[15]

[f] A. Albert, *Biochem. J.* **54,** 646, 1953.

[g] Ogston.[17]

[h] Sugar group.

[i] 5′ Deoxynucleotides: R. O. Hurst, A. M. Marko and G. C. Butler, *J. Biol. Chem.* **204,** 847, 1953.

[j] Presumably same as riboside.

[k] Cavalieri *et al.*[16]

[l] Not measured but presumably similar to base.

[m] Shugar and Fox.[18]

II. The Separation of Bases and Nucleosides

1. Ionic Properties

All purine and pyrimidine bases have at least one group capable of forming an ion. Most have more than one, and can ionize, under proper conditions, to form either anions or cations (see Table I). The ribosides have properties very similar to those of the bases, since the very weakly acid ribose residues do not interfere (except in the case of adenosine, where ribos replaces the only easily dissociable hydrogen).

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[12] P. A. Levene and L. W. Bass, "The Nucleic Acids." Chemical Catalog Co., New York, 1931.

[13] W. E. Cohn, unpublished observations.

[14] R. A. Alberty, R. M. Smith, and R. M. Bock, *J. Biol. Chem.* **193,** 425 (1951).

[15] H. F. W. Taylor, *J. Chem. Soc.* **1948,** 765.

[16] L. Cavalieri, S. E. Kerr, and A. Angelos, *J. Am. Chem. Soc.* **73,** 2567 (1951).

[17] A. G. Ogston, *J. Chem. Soc.* **1935,** 1376.

[18] D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta* **9,** 199 (1952).

In acid solution, the amino groups of guanine, adenine, and cytosine ar cationic, with pK values increasing in that order (see Table I). Uracil an thymine and their ribosides have no amino groups and are not sorbed to a appreciable degree by cation exchangers in the hydrogen form. Hypoxan thine and xanthine and their ribosides, also not cationic, exhibit a smal degree of retention on cation exchangers.[13] This retention may be ascribe to a nonpolar attraction, seemingly general among the purines, giving them a higher distribution coefficient than pyrimidines of equal pK values. Such a family difference, noted in the nucleotides,[19] may explain the greater retention of adenosine over cytidine found on the carboxylic resin IRC-50,[20] where ionic considerations alone would predict the reverse.

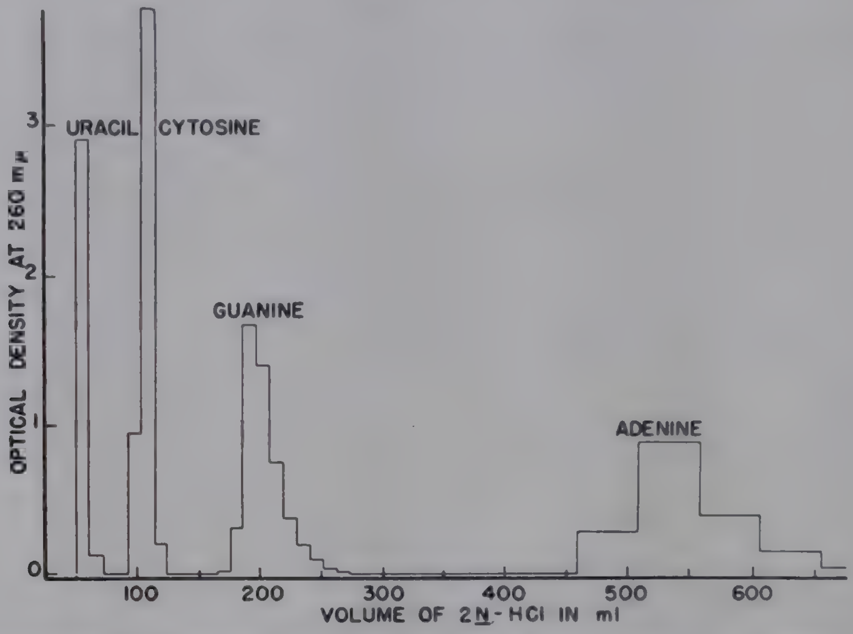

FIG. 2. Separation of purine and pyrimidine bases by cation exchange in an acid system.[21]
Exchanger: Dowex-50-H$^+$, ca. 300 mesh, 8.1 cm. × 0.74 cm.^{2}.
Solution: 2 *N* HCl, 0.6 ml./min.
Sorbed material: 0.5–1.0 mg. of each base in 7.5 ml. 2 *N* HCl. (Larger volumes of more dilute acid may be used.)

2. Cation Exchange

In Fig. 2 is indicated the separation of the four bases of ribonucleic acid (such as could be derived by formic acid[22] or perchloric acid hydrolysis[23]) by cation exchange, utilizing ionic-strength adjustment without any charge adjustment (5-methylcytosine follows cytosine by a very slight margin, whereas cytidine precedes it by a factor of 3.4[13]). Uridine and cytidine have been separated from pyridine or enzymic digests by sorption of the latter on a sulfonic acid resin, followed by displacement with ammonia or pyridine.[24, 25] The possibilities of a separation of the bases by charge adjustment (e.g., by increasing pH in a sodium or ammonium cycle, as Stein and Moore[4] have done for amino acids) has not been tried on the bases, but Reichard and Estborn[26] have utilized this principle for the preparation of four (N^{15}-labeled) deoxynucleosides in an ammonium cycle. Since adenosine was not present in their mixture (the enzyme preparation having converted it to deoxyinosine), no information is at hand as to how well adenosine and guanosine might separate in such a system. Extensive hydrolysis at the acid-sensitive glycosidic linkage of the purines has accompanied other experiments along this line.[20] Such hydrolysis has also been observed with the purine ribonucleotides in the hydrogen cycle.[19, 21]

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[19] W. E. Cohn, *J. Am. Chem. Soc.* **72,** 1471 (1950).
[20] W. Andersen, C. A. Dekker, and A. R. Todd, *J. Chem. Soc.* **1952,** 2721.
[21] W. E. Cohn, *Science* **109,** 377 (1949).
[22] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176,** 715 (1948).
[23] A. Marshak and H. J. Vogel, *J. Biol. Chem.* **189,** 597 (1951).

3. Anion Exchange

a. General

The anionic properties of the bases reside chiefly in the keto groups, which can enolize and thus develop ionizable hydrogens. While the hydroxyl groups of ribose are also weak acids, it has seldom been necessary to resort to the high pH required to ionize them. The attachment of ribose to the purine and pyrimidine bases does not interfere with the acidic group (except in the case of adenine), so that the behavior of bases and ribosides is again very similar. The absence of glycosidic lability in alkaline regions make the anion-exchange method preferable to cation exchange. In addition, it is possible to exploit the method of elution by charge adjustment, thus removing the substances by very dilute (e.g., 0.01 M) solutions buffered in the region of the pK's involved instead of the concentrated acid solutions employed in cation-exchange separations.

b. Bases

The five bases normally considered as constituents of nucleic acid have enolic pK's in the descending order cytosine, adenine, thymine, guanine, and uracil. The order of elution by NH_4OH–NH_4Cl buffers (Fig. 3) shows two aberrations from this sequence: adenine follows guanine and uracil precedes thymine. In the latter case, the additional methyl group may be responsible for the higher distribution coefficient.

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[24] D. T. Elmore, *Nature* **161,** 931 (1948); *J. Chem. Soc.* **1950,** 2084.

[25] R. J. C. Harris and J. F. Thomas, *Nature* **161,** 931 (1948); *J. Chem. Soc.* **1948,** 1936.

[26] P. Reichard and B. Estborn, *Acta Chem. Scand.* **4,** 1047 (1950).

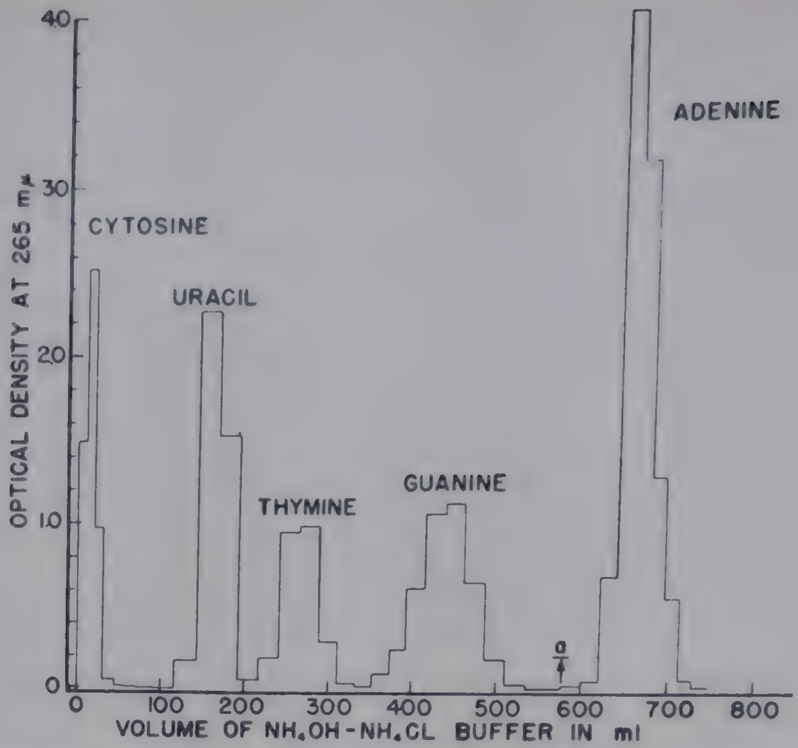

FIG. 3. Separation of purine and pyrimidine bases by anion exchange in a chloride system.[21]

Exchanger: Dowex-1-Cl$^-$, ca. 300 mesh, 8.5 cm. × 0.74 cm.^{2}.

Solution: 0.2 *M* NH_4OH + 0.025 *M* NH_4Cl, pH 10.6 (at *a*, pH → 10.0, Cl^- → 0.1 *M*); 0.25 ml./min.

Sorbed material: 1–2 mg. of each base in eluting buffer.

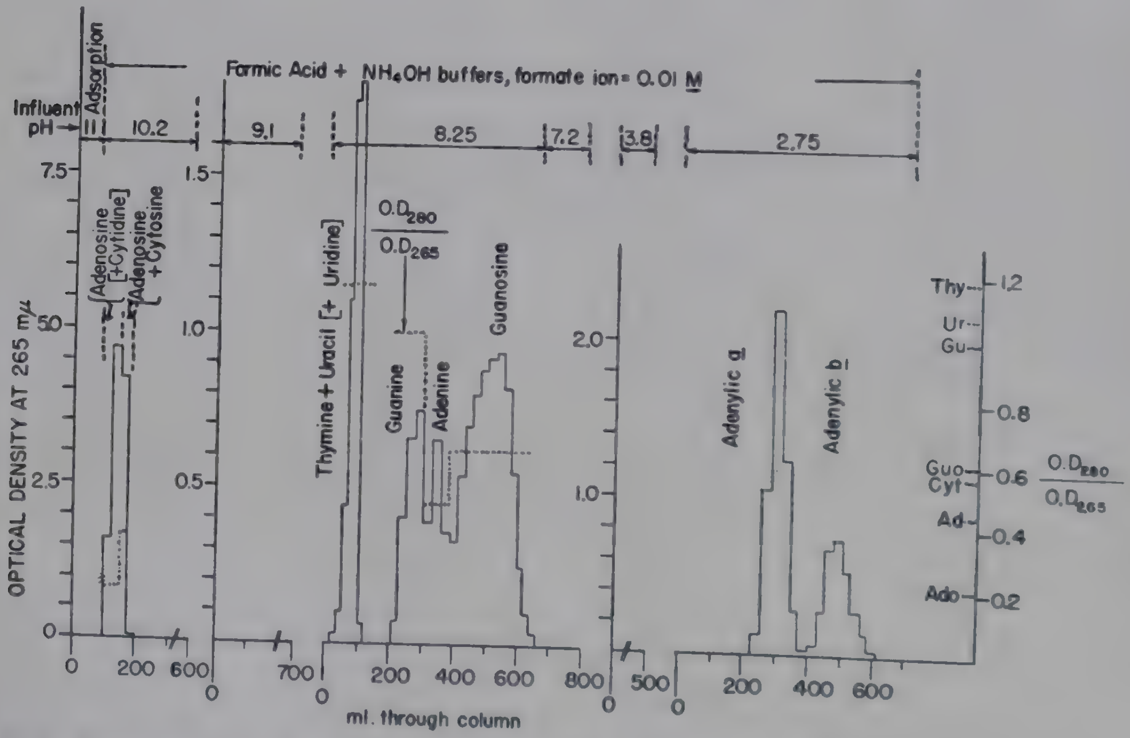

FIG. 4. Separation of bases, ribosides, and nucleotides by anion exchange in a formate system.[19]

Exchanger: Dowex-1-formate, 13 cm. × 0.74 cm.^{2}.

Solutions: as shown, 0.5 ml./min.

Sorbed material: as shown. Substances in brackets were not included in this separation but were examined separately. The dotted line indicates the method of identification by spectrophotometric absorption ratios.

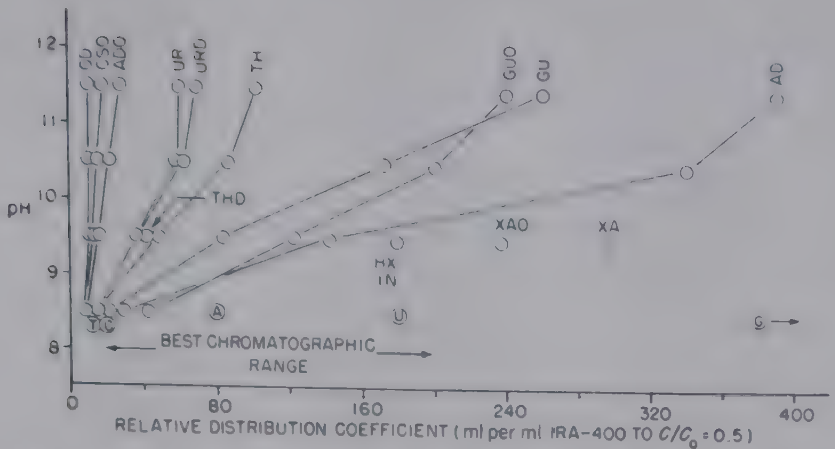

FIG. 5. Relative distribution coefficients (ml. effluent per ml. exchanger to $C/C_0 = 0.5$) of bases and ribosides as a function of pH in 0.01 M Cl^-, as derived from breakthrough experiments.[13]

Exchanger: 1 ml. IRA-400.

(Circled letters T, C, A, U, and G are taken from Fig. 20, to show the influence of borate ion on thymidine (no effect), cytidine, adenosine, uridine, and guanosine, respectively.)

c. *Ribosides*

The similarity in the behavior of the ribosides is shown in Fig. 4, in which a mixture of bases and ribosides has been chromatographed. This method has been applied to the large-scale separation of the deoxynucleosides[20] for preparative purposes.

A summary of breakthrough sorption experiments on bases and nucleosides, for the purpose of determining relative distribution coefficients, is given in Fig. 5.

III. Separation of Nucleotides

1. MONONUCLEOTIDES

a. *Ionic Properties*

The nucleotides differ markedly in ion-exchange characteristics from the bases and ribosides by virtue of the presence of the strongly acidic phosphoryl groups. Phosphate esters are stronger acids than inorganic orthophosphate itself, with pK values of about 1 and 6 for the first and second dissociation, respectively, as against 2 and 7 for orthophosphate. The difference in secondary ionization constants makes possible the easy separation of inorganic phosphate from phosphoric acid esters in the region of pH 6 (see Sect. III.1.*d*).

b. *Distribution Coefficients*

Among the nucleotides, anionic behavior is predominantly a function of the phosphate group, but this is modified by the ionic and nonpolar properties of the nucleoside residues. An assessment of the total charge due to the ionization of phosphate and of base groups as a function of pH is plotted Fig. 6. The order of elution predicted from these considerations is subje to modification by the greater attraction of the exchanger for purines ov pyrimidines mentioned earlier. Thus, in order to superimpose the separ tions found (Fig. 7) on those predicted solely from the net-charge criterio (Fig. 6), it must be assumed that the purine compounds are about thre times as strongly sorbed as the pyrimidines.[19] This factor remains fairl constant over a wide range of pH (and hence of net charge), thus indicat ing that it probably has to do with intrinsic properties of the solutes—whic differ only in the base constituents—and not with ionic properties which are pH dependent.

The variation of elution position with pH (Fig. 7) is of some interest particularly in view of the increasing use of volatile salts for elution rather than mineral acids. At neutral pH, similar purine nucleotides appear together and similar pyrimidine nucleotides appear together, the latter separated (by the factor of about 3) from the former. It may be concluded that the separation of each pair at more acid pH is due to the development of a cationic group (the ammonium group), causing adenylic to precede guanylic acid (pK 3.7 vs. 2.3) and cytidylic to precede uridylic acid (pK 4.2 vs.

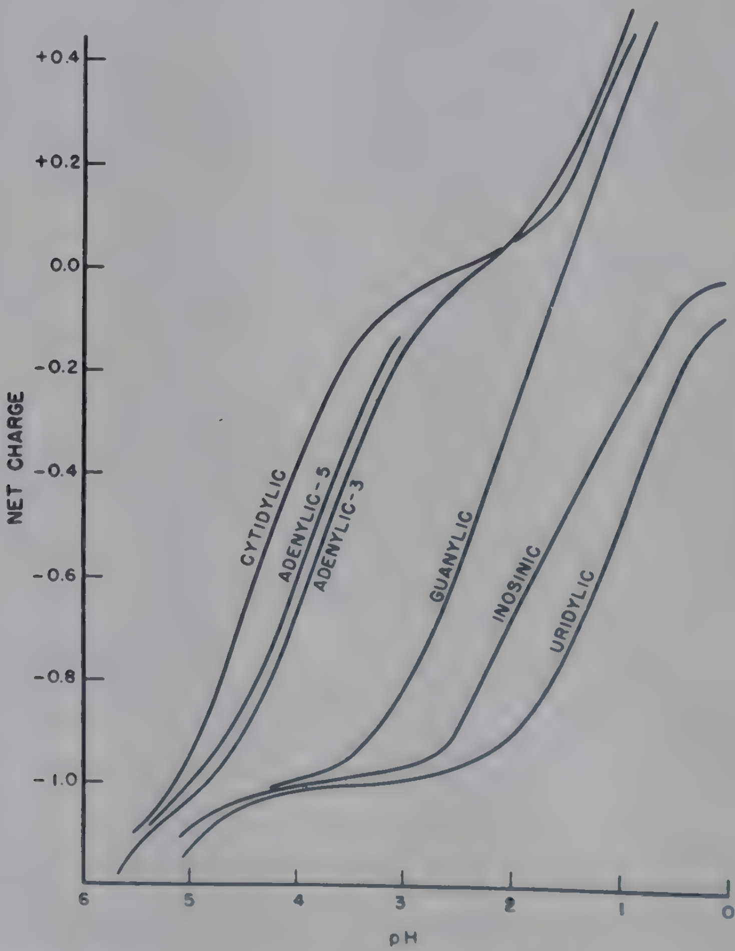

Fig. 6. Net charge per molecule of ribonucleotide as a function of pH, calculated[19] from the pK values quoted in Levene and Bass.[12]

none). Since uridylic acid has no cationic group, we express the data in terms of uridylic acid elution (the parallelism of ionosinic acid should be noted). The relative positions of cytidylic and of adenylic acids are maintained at all pH values; those of uridylic and guanylic acids become reversed at low pH due to the development of a charge upon the latter. The same is true of adenylic acid and its derivative, inosinic acid.

c. Elution with Dilute HCl

A separation of four purified ribose nucleotides by dilute HCl at pH 2.5 is shown in Fig. 8.[19] The mixture was sorbed from dilute ammoniacal solution, with total chloride concentration less than 0.01 *M*, followed by 0.01 *M* NH_4Cl until the effluent pH fell to 7. (This NH_4Cl wash removes any bicarbonate present and thus prevents CO_2 formation when the acid is started, as well as eliminating a long delay until all ampholytes on the resin are neutralized; it is generally advisable when proceeding from alkaline to acid solutions.) The order predicted from Fig. 6 is not observed with respect to the uridylic–guanylic acid sequence, nor with respect to the distance between cytidylic and adenylic acids; the aberrations are accounted for by the factor discussed and are set forth in Fig. 7. Fig. 8 also illustrates the use of specific ultraviolet absorption characteristics to follow the course of a separation and the extent to which the system follows the Mayer and Tompkins equation,[8] derived upon the assumption that equilibrium between exchanger and solute is achieved throughout the elution sequence.

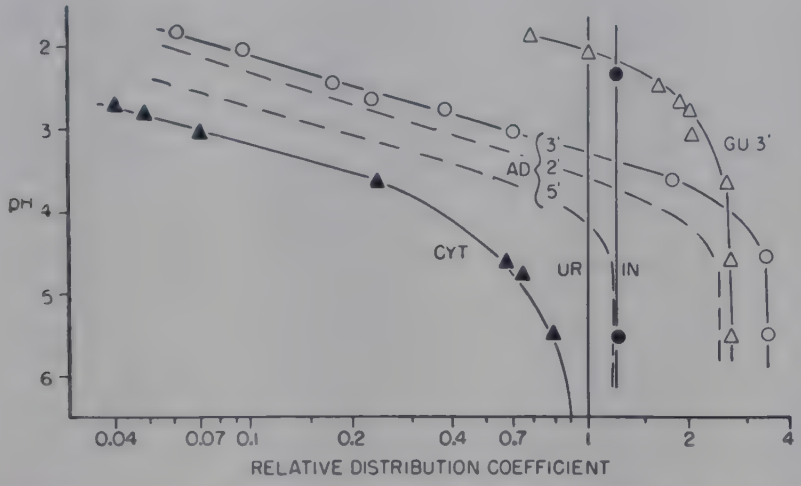

FIG. 7. Observed relative distribution coefficients (positions of elution peaks) of ribonucleotides as functions of pH, derived from column chromatography (see Fig. 8).

The separation of the deoxynucleotides (Fig. 9[27-29]) follows a similar pattern with thymidylic taking the place of uridylic acid. From the deoxy-

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[27] E. Volkin, J. X. Khym, and W. E. Cohn, *J. Am. Chem. Soc.* **73,** 1533 (1951).

[28] R. L. Sinsheimer and J. F. Koerner, *Science* **114,** 42 (1951).

[29] R. O. Hurst, J. A. Little, and G. C. Butler, *J. Biol. Chem.* **188,** 705 (1951).

cytidylic acid peak, deoxy-5-methylcytidylic acid can be separated [s Sect. III.1.*e*(3)].

d. Use of Higher pH; the Separation of Inorganic Phosphate and Nonnucle tide Phosphoric Acid Esters

Elution of nucleotides by dilute acids involves both principles of elutio mentioned earlier, charge adjustment (pH dependent) and ionic strengt adjustment (chloride-ion dependent). With increase in pH it is also necessary to increase the chloride ion in order to maintain a reasonable rate o elution; thus at pH 5.6, a chloride concentration of about 0.02 *M* is necessary to maintain rates of elution comparable to those indicated for 0.00 *N* HCl in Fig. 8. Under these conditions, cytidylic and uridylic acids appear together, as do guanylic and adenylic acids (see Fig. 7). Inorganic phosphate, which appears in the adenylic acid region at pH 2–3, precedes the pyrimidine nucleotides by a wide margin at pH 6. Creatine phosphate and sugar phosphates behave similarly in the acid region but are separable from inorganic phosphate in the neutral region by virtue of the lower sec-

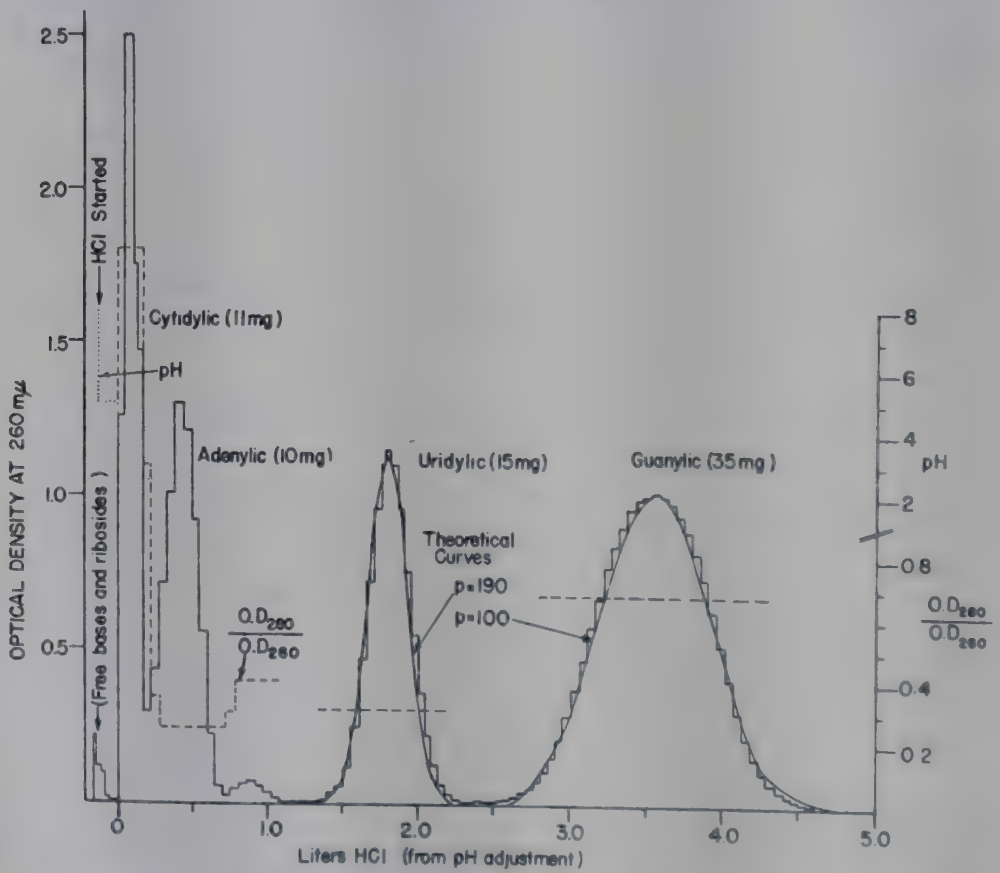

FIG. 8. Separation of ribonucleotides by anion exchange in a chloride system showing conformity with calculated theoretical curves (see text).[19]

Exchanger: Dowex-2-chloride, 12.5 cm. × 0.94 cm.².
Solution: 0.003 *N* HCl, 0.8 ml./min.
Sorbed materials: purified nucleotides, in amounts shown.

ondary pK of phosphoric acid esters. The data[13] on the separation of these substances are summarized in Fig. 10.

e. Isomeric Nucleotides

(1) *Choice of Anion.* Other acids and their salts may be used in place of chloride. In general, those anions with a greater affinity than chloride for the exchanger (e.g., Br^-, SO_4^{--}) will effect a given rate of elution with lower ionic strength or lower hydrogen-ion concentration or both; those with a lesser affinity (e.g., formate, acetate) will require a lower pH or a higher anion concentration. The effectiveness of a given anion in replacing a specific solute will depend upon its affinity for the exchanger, which is a function of pK, and upon intrinsic properties. Formate has received much attention,[11, 19] partly because of the ease of obtaining solutions of stable pH in the region of 3-4 and partly because of the ease with which it and its

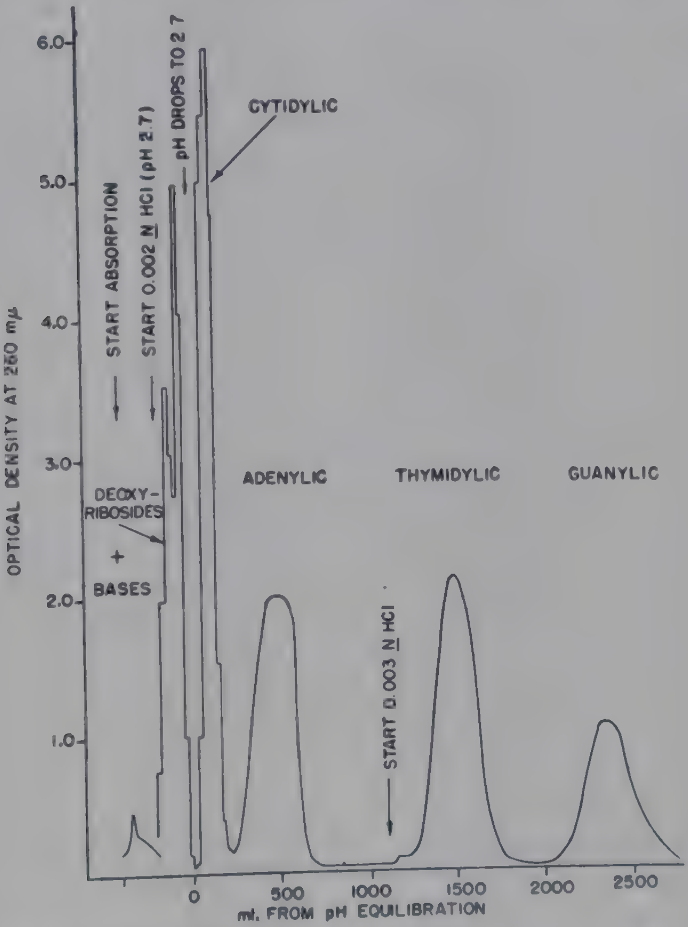

FIG. 9. Separation of deoxyribonucleotides by anion exchange.[27]
Exchanger: Dowex-1-chloride, 200–400 mesh, 8 cm. × 0.72 cm.².
Eluting solution: HCl as shown, 1 ml./min.
Sorbed material: mixed deoxynucleotides isolated by preliminary ion exchange from 150 mg. DNA digested with deoxyribonuclease followed by intestinal phosphatase plus arsenate (method of Klein and Thannhauser used by Volkin *et al.*[27] and Sinsheimer and Koerner[28]).

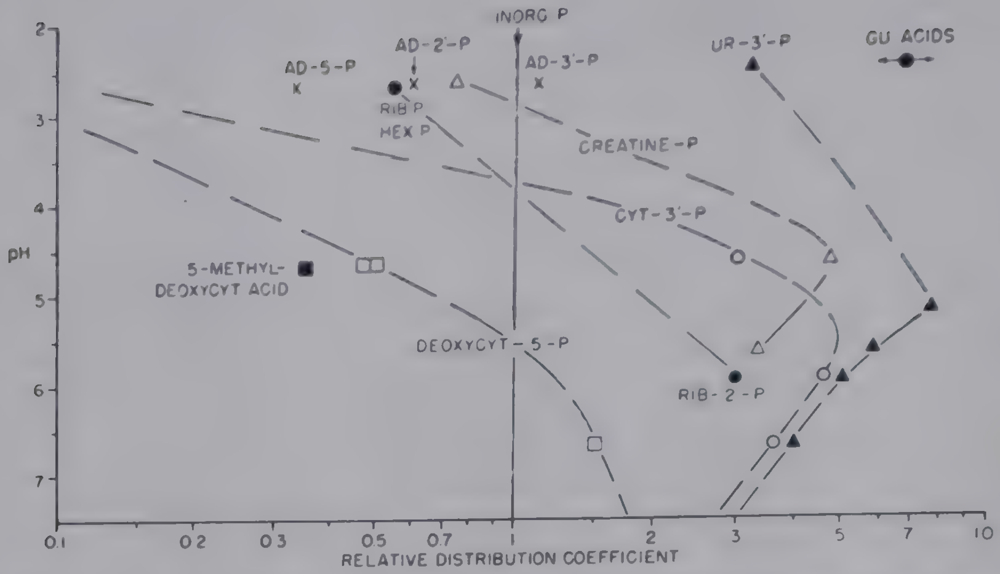

FIG. 10. Relative distribution coefficients of various nucleotides and organic phosphates relative to inorganic phosphate as a function of pH, derived from anion-exchange chromatography[13] (see Fig. 7).

ammonium salt can be volatilized. Acetate was used in the first ion-exchange separations of the 2′ and 3′ nucleotide isomers.[19, 30-32] On the other hand, the dilute HCl solution has an advantage, if subsequent concentration by resorption and re-elution is considered,[19, 33] because of the low ionic strength; the more concentrated formate or acetate solutions must usually be diluted before resorption, if such is indicated.

(2) *pK's of Isomeric Nucleotides.* The 5′-, 2′-, and 3′-phosphate isomers of the various ribonucleosides appear in the order named upon elution under the proper conditions for separation. It would appear, compositional factors being the same, that the pK values of the phosphate and amino groups, which differ slightly among the isomers, might be responsible for the separations to be presented. These values are not known for all sets of isomeric nucleotides; those so far reported are collected in Table II. It will be seen that, in general, they vary in magnitude and in direction as one might infer from the order of elution, and hence may properly be held to be responsible for the separations.

(3) *Cytidylic Acids.* The cytidylic acid isomers are eluted rapidly with 0.001 *N* HCl solution and with 0.01 *N* formic acid;[40] hence, a pH of 3 or

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[30] J. X. Khym and W. E. Cohn, unpublished observations.

[31] W. E. Cohn, *J. Am. Chem. Soc.* **72,** 2811 (1950).

[32] H. S. Loring, N. G. Luthy, H. W. Bortner, and L. W. Levy, *J. Am. Chem. Soc.* **72,** 2811 (1950).

[33] W. E. Cohn, *J. Am. Chem. Soc.* **71,** 2275 (1949).

[34] W. E. Cohn, *J. Cellular Comp. Physiol.* **38,** Suppl. 1, 21 (1951).

[35] L. F. Cavalieri, *J. Am. Chem. Soc.* **74,** 5804 (1952); **75,** 5268 (1953).

[36] H. Wassermeyer, *Z. physiol. Chem.*, **179,** 238 (1928); see Levene and Bass,[12] p. 231.

[37] P. A. Levene and H. S. Simms, *J. Biol. Chem.* **65,** 519 (1925); **70,** 327 (1926); see Levene and Bass,[12] pp. 223, 228.

TABLE II

pK VALUES OF ISOMERIC NUCLEOTIDES AND RELATED SUBSTANCES

	NH_2 Group	Secondary Phosphate
Adenine	4.12,[a] 4.16[b]	—
Adenosine	3.63,[a] 3.4[c]	—
Adenosine-5′-phosphate	3.74,[a] 3.89,[b] 3.8[d], 4.4[k]	6.05,[a] 6.48,[b] 6.2[d], 6.4[k]
Adenosine-2′-phosphate	3.80,[a] 3.79[b]	6.15,[a] 6.21[b]
Adenosine-3′-phosphate	3.65,[a] 3.56,[b] 3.70[e]	5.88,[a] 6.06,[b] 6.01[e]
H_3PO_4	—	6.86[b]
-Glycerophosphate	—	6.45[b]
Glucose-1-phosphate	—	6.14[b]
Cytosine	4.45,[f] 4.6[g]	—
5-Methylcytosine	4.6,[f] 4.8[g]	—
Cytidine	4.11,[h] 4.15,[g] 4.25[i,h]	—
5-Methylcytidylic acid	4.4[g]	—
Cytidine-5′-phosphate	4.44,[h] 4.45[i,g], 4.6[k]	6.6[k]
Cytidine-2′-phosphate	4.30,[j,h,g] 4.36[c]	6.2[j]
Cytidine-3′-phosphate	4.3,[j] 4.28,[c,g,e] 4.16[h]	6.0[j,e]

[a] Alberty *et al.*[14] (25°, 0.15 *M* NaCl).

[b] Kuna, M., *U.S. Atomic Energy Comm. Rept.* **ORNL-318** (May 10, 1949); quoted by Cohn.[34]

[c] Cavalieri.[35]

[d] Wassermeyer[36] (see Levene and Bass,[12] p. 231).

[e] Levene and Simms[37] (see Levene and Bass,[12] pp. 223, 228).

[f] Shugar and Fox[18] (spectrophotometric).

[g] Cohn[13] (spectrophotometric).

[h] Fox *et al.*[38] (spectrophotometric).

[i] Deoxy compound.

[j] Loring *et al.*[39]

[k] For deoxy 5′ nucleotides: R. O. Hurst, A. M. Marko, and G. C. Butler, *J. Biol. Chem.* **204**, 842 (1953)

higher is necessary for their separation. Formate and acetate are more manageable from the standpoint of pH control and buffer capacity in this range and give good separations of the 5′-, 2′-, and 3′-phosphates of cytidine, in that order. Attempts to prepare large amounts with 0.05–0.1 *M* formic acid have given incomplete separation, apparently owing to an overloading of the columns as well as to an excessively rapid elution with the higher acid concentration.[39] Deoxycytidylic acid (also a 5′ ester) is inseparable from the ribose analogue under these conditions, but can be separated with the aid of borate complexing (see Sect. IV.2.*c*). Deoxy-5-methylcytidylic acid precedes the 5′ nucleotide(s) by a small margin. Fig. 11a[31] demonstrates the separation of these substances with acetate buffers. This experiment is similar to that in which were discovered the existence of 5-methylcytidylic acid[41] in deoxycytidylic acid and the existence of two isomers (2′ and 3′)

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[38] J. J. Fox, L. F. Cavalieri, and N. Chang, *J. Am. Chem. Soc.* **75,** 4315 (1953).

[39] H. S. Loring, H. W. Bortner, L. W. Levy, and M. L. Hammell, *J. Biol. Chem.* **196,** 807 (1952).

[40] W. E. Cohn and E. Volkin, *Nature* **167,** 483 (1951).

[41] W. E. Cohn, *J. Am. Chem. Soc.* **73,** 1539 (1951).

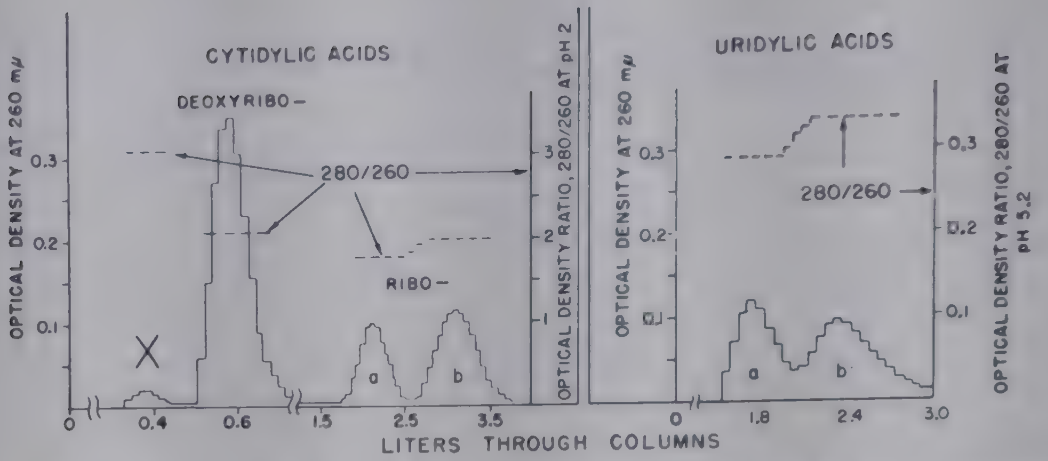

FIG. 11. Separation of (a) cytidylic and (b) uridylic acids by anion exchange.[3
Exchanger: Dowex-1-acetate (a), -formate (b), 200–400 mesh, (a) 11.3 cm.
0.86 cm.^{2}, (b) 9.5 cm. × 0.76 cm.^{2}.
Solutions: (a) 0.025 M HAc + 0.025 M NaAc, (b) 0.04 M Na formate + 0.00
M formic acid.
Sorbed materials: (a) crude deoxycytidylic acid fraction[27, 41] (see Fig. 9), co
taining deoxy-5-methylcytidylic acid (shown as "X"), plus cytidylic acids *a* an
b (2′ and 3′); (b) uridylic acids *a* and *b* (2′ and 3′).

of cytidylic acid. Similar separations are achieved in 0.01 N formic aci
In Fig. 11b is demonstrated the separation of the 2′ and 3′ isomers o
uridylic acid in a formate system. These are not well separated in aci
systems.

(4) *Adenylic and Inosinic Acids.* The 5′-, 2′-, and 3′-phosphate isomer of adenosine are easily separable by 0.002 N HCl[19, 27, 33] or by 0.1 M formi acid.[34, 40] (A separation at pH 5.5 in a chloride system has also been described.[34] At this pH, the inosinic acid isomers, included in the experiment precede the corresponding adenylic acids just as do the uridylic acids see Fig. 12.) Fig. 13 shows the separation of a partially deaminated mixture of the three adenylic acids by formic acid.

(5) *Uridylic Acids.* It will be noted that the inosinic acids not only follow the adenylic acids at a considerable distance but are not well separated by formic acid. The uridylic acids show the same behavior (see Fig. 5[19]). Hence, in practice, resort has been made, for the separation of uridylic acid isomers after the removal of the cytidylic and adenylic acids, to buffer systems of higher pH and ionic strength (see Fig. 11). This also avoids entangling the uridylic acids with the guanylic acids; it will be recalled (see Fig. 7) that these two groups are not well separated at low pH.

(6) *Guanylic Acids.* The guanylic acid isomers separate well at all pH values tested. Separations at pH 2.5 (HCl) and 5 (formate) have been reported,[19, 40] both as part of the analysis of an alkaline digest of PNA which includes the four isomeric pairs of nucleotides.

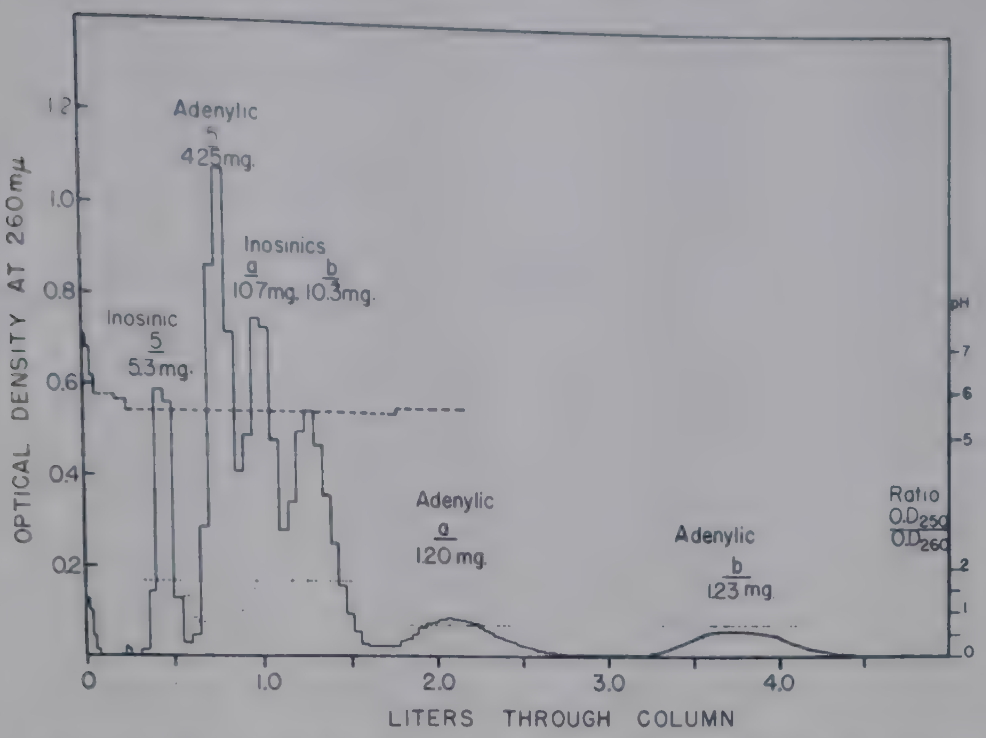

FIG. 12. Separation of three adenylic acids and the corresponding inosinic acids by anion exchange at pH 5.5 in a chloride system.[34]

Exchanger: Dowex-1-chloride, 250–500 mesh, 9.5 cm. × 0.9 cm.².

Solution: 0.02 *M* NaCl in 0.01 *M* acetate buffer, pH 5.5, at 1.2 ml./min.

Sorbed material: commercial adenylic acids (ca. 10 mg. each), partially deaminated with nitrous acid.

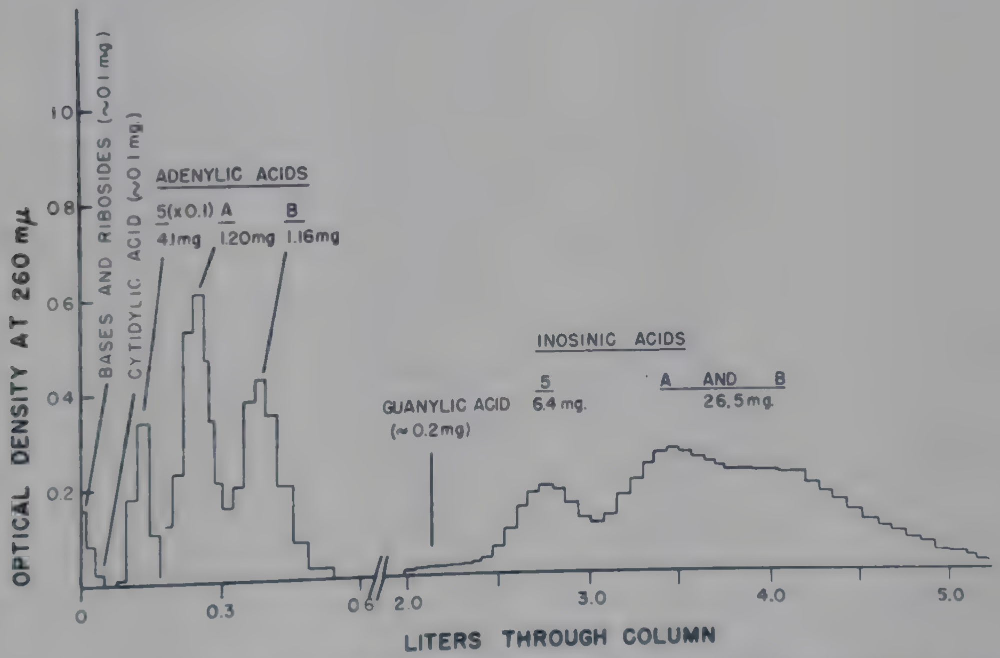

FIG. 13. Separation of three adenylic acids and the corresponding inosinic acids by anion exchange with formic acid.[13]

Exchanger: Dowex-1-formate, 250–500 mesh, 13 cm. × 0.74 cm.².

Solution: 0.125 *M* formic acid, 1.5 ml./min.

Sorbed material: same as in Fig. 12.

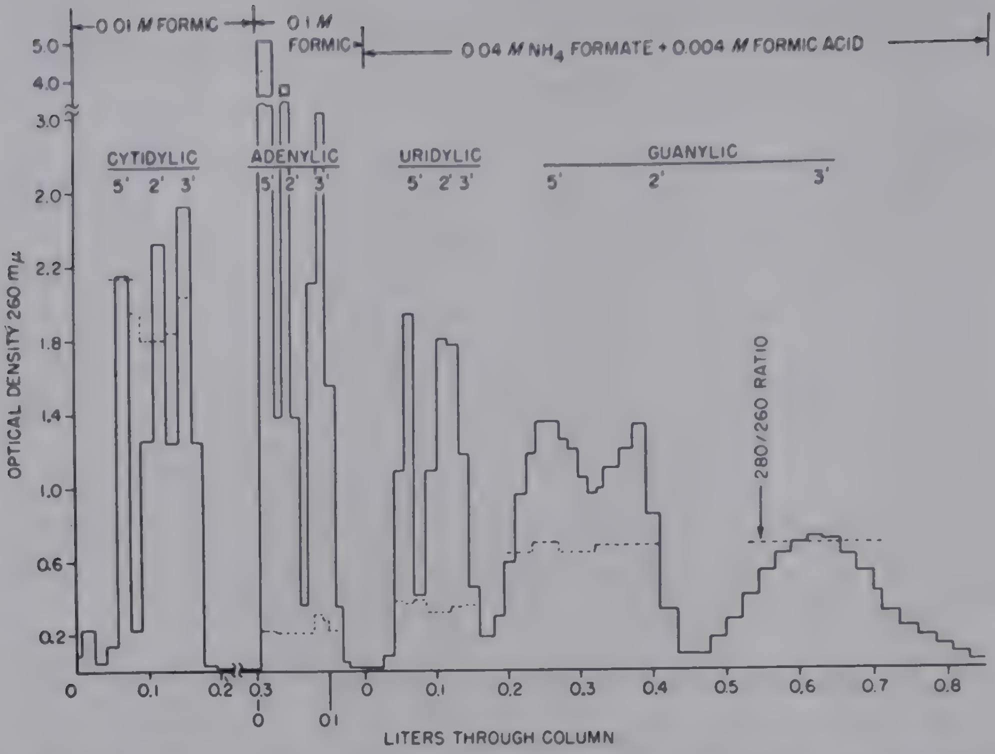

FIG. 14. Separation of the isomeric 2′, 3′, and 5′ nucleotides of ribonucleic acid by anion exchange in a formate system.[13]

Exchanger: Dowex-1(× 2%)-formate, 400 mesh, 5 cm. × 0.82 cm.^{2}.
Solution: Formic acid, ammonium formate as shown.
Sorbed material: Combined alkaline digest and 5′ nucleotides (diesterase digest) prepared as follows: 25 mg. calf liver PNA + 1 ml. 0.5 *N* NaOH, 37°C., 18 hr.; diluted to 50 ml.; added 2 mg. each of 5′ nucleotides (prepared as described in Fig. 16) + 0.5 meq. NH_4 formate + NH_4OH to 0.1 *M*.

(7) *Application to Digests.* A demonstration of the separation of the 5′, 2′, and 3′ isomers of all four ribonucleotides is shown in Fig. 14.[13] It has been customary, in order to save time, to double the concentration of formate and lower the pH with formic acid after removal of the uridylic acids (see Fig. 15).

The use of this sytem in the analysis of unknown digests and of the usual alkaline digest is shown in Fig. 15.[40] This is the actual experiment in which the 5′ nucleotides were first isolated from an enzyme digest of PNA (See also Cohn and Volkin[42] and Fig. 16).

2. POLYPHOSPHONUCLEOSIDES

a. Diphosphates

The mixed 2′,5′-, and 3′,5′-diphosphates of cytidine and uridine have been recovered by ion exchange from digests of PNA with rattlesnake venom freed of 5′ monoesterase.[42, 43] These digests also contain the 5′ nucleotides of all four bases, and 3′ pyrimidine nucleotides, and all four

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[42] W. E. Cohn and E. Volkin, *Arch. Biochem. and Biophys.* **35,** 465 (1952).
[43] W. E. Cohn and E. Volkin, *J. Biol. Chem.* **203,** 319 (1953).

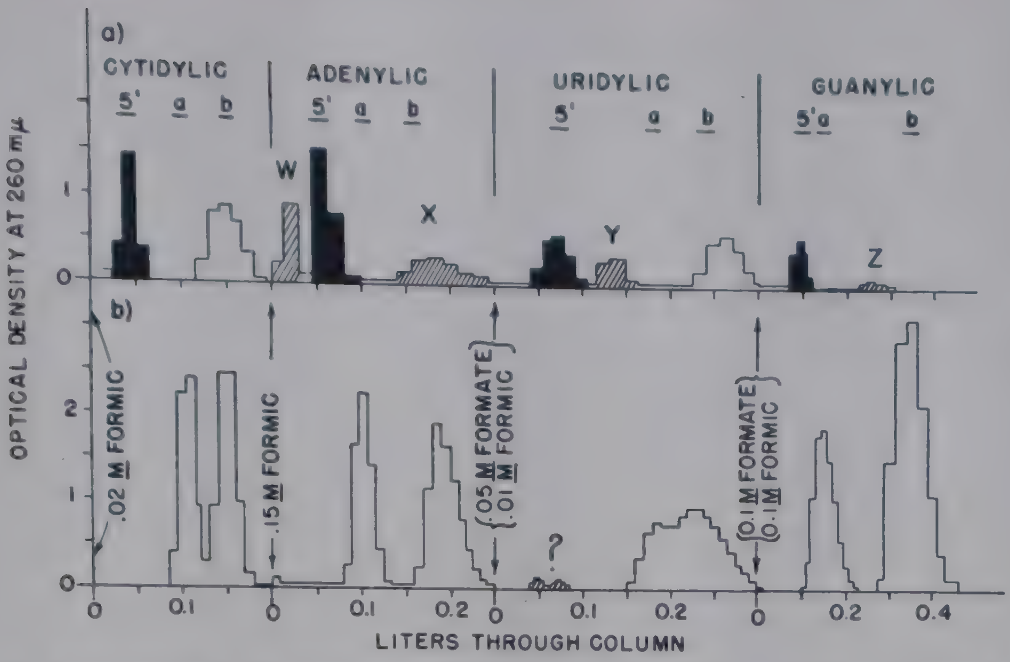

FIG. 15. Analysis of (a) enzymic, (b) alkaline digest of calf liver PNA by anion exchange.[40]

Exchanger: Dowex-1-formate, 200–400 mesh, 7 cm. × 0.88 cm.^{2}.

Solutions: as shown.

Sorbed material: (a) 100 mg. (20 ml.) PNA + 7 mg. ribonuclease digested 5 hr., titrated with (112 μeq.) NaOH to hold at pH 7.2; diluted to 25 ml.; 12 ml. + 0.8 ml. 0.1 M arsenate + 6 ml. (20 mg.) intestinal phosphatase; digested 90 min., titrated with 1.5 ml. 0.05 M NaOH to hold at pH 8.5; diluted to 25 ml.; acidified to pH 2.9 with formic acid; centrifuged. Supernatant plus NH_4OH sorbed on column. (b) 4 ml. same PNA solution + 2 meq. NaOH; digested 15 hr. at 37°C.; diluted to 25 ml. with 2 meq. NH_4 formate + 2 meq. NH_4OH; sorbed on same column subsequently.

nucleosides, the adenosine appearing as inosine.[43] The recovery of these components and the mode of isolation and discovery of the diphosphates is shown in Fig. 16.

The positions of the two diphosphates are of some interest. It might be expected that the differentiation in ion-exchange behavior of these substances would depend on the bases to the same extent as in the monophosphates and thus bring about the same order of elution as is observed in the monophosphates in pH ranges adjusted to offset the increased number of acid groups. That such is the case is indicated by the order of the two pyrimidine diphosphates in Fig. 16 and by the fact that adenosine-5′-pyrophosphate (ADP) comes between them (see Fig. 17, in which the peak positions of several substances examined independently are indicated by arrows). The position of inorganic pyrophosphate between ADP and uridine diphosphate is also similar to orthosphosphate in the monophosphate series. It is also apparent that the diphosphates as a group are sufficiently strongly bound to the anion-exchange resin to follow the last of the monophosphates, overlapping only slightly with guanylic (and inosinic) acid. (The behavior of riboflavin phosphate is ascribed to its added nonpolar affinity.) Th factors discussed in Section III.1.*b* may cause some shifting of the order elution in other pH ranges.

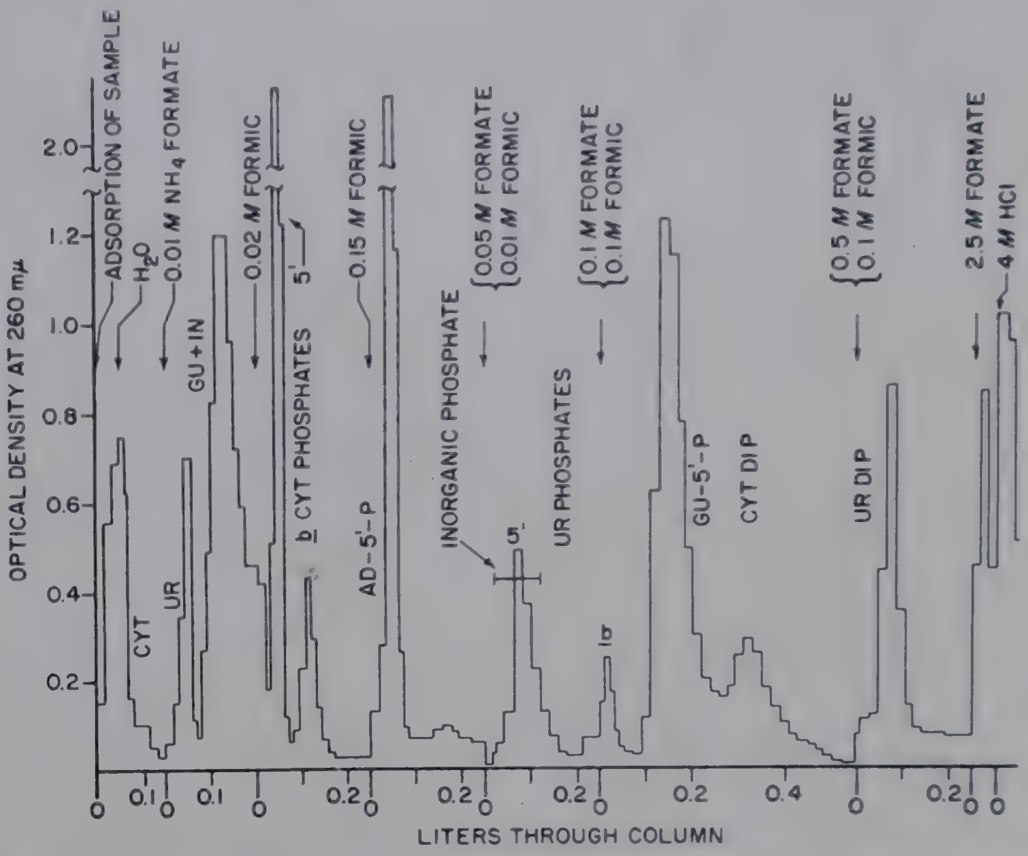

FIG. 16. Analysis of snake venom diesterase digest of PNA by anion exchange.[43]
Exchanger: Dowex-1-formate, 400 mesh, 5.8 cm. × 0.9 cm.^{2}.
Solutions: as shown, at 0.5 ml./min.
Sorbed material: 16 mg. calf liver PNA + 1 ml. 0.05 *M* $MgCl_2$ + 1 ml. snake venom diesterase preparation (rattlesnake venom freed of 5′ monoesterase by the method of Butler[29]), total volume 5 ml.; pH 8.6 maintained by addition of 0.02 *N* NaOH for 7 hr. at 25°C.; diluted to 100 ml. with 1 meq. NH_4OH; sorbed.

b. Polyphosphates

Similarly, ATP follows uridine-3′,5′-diphosphate (and also uridine-5′-pyrophosphate[44]), and it has been demonstrated[44] that the entire group of triphosphorylated nucleosides may be separated from the diphosphorylated ones as well as the latter are from the monophosphates. The great difference in affinity has been exploited to effect rapid separations of adenosine-5′-phosphate from ADP and ATP.[45]

Finally, the position of hexametaphosphate may be noted in Fig. 17 as further evidence that gross affinity increases with the degree of polyvalency of the ion involved.

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[44] R. B. Hurlbert, and V. R. Potter, *J. Biol. Chem.* **209,** 1 (1954).

[45] W. E. Cohn and C. E. Carter, *J. Am. Chem. Soc.* **72,** 4273 (1950).

3. Polynucleotides

a. Ionic Properties and Molecular Size

The polynucleotides derived from nucleic acid by the action of enzymes, e.g., deoxyribonuclease on DNA[46, 47] or ribonuclease on PNA,[48, 49] or by brief acid treatment[50] can be separated by ion-exchange, if consideration is given to their greater size and charge. These properties influence the rates of diffusion and the distribution coefficients. The latter must be compensated for, as in the simpler polyphosphates, by increased acidity or ionic strength. To accommodate the large size, it has been found advantageous,[48, 49] in working with polynucleotides, to utilize anion exchangers of a lower degree of cross-linking (divinylbenzene content) than the so-called "standard" resins although successful separations have been reported using

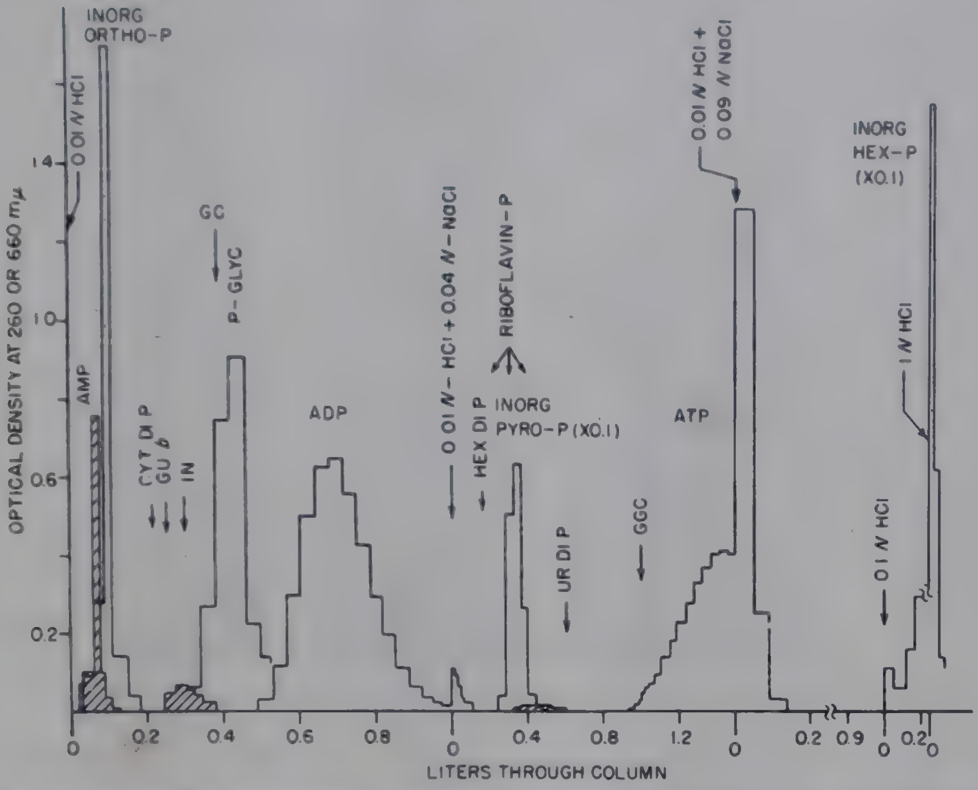

Fig. 17. Separation of various phosphates by anion exchange in chloride system.[13]
Exchanger: Dowex-1-chloride, 200–400 mesh, 10 cm. × 0.76 cm.^{2}.
Solution: HCl + NaCl as shown, 1 ml./min.
Sorbed material: 2–5 mg. of each substance shown. (Substances with peak positions indicated by arrows only were not included in the separation shown, but were examined on similar columns or the same column independently.)

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[46] R. L. Sinsheimer and J. F. Koerner, *J. Am. Chem. Soc.* **74**, 283 (1952).

[47] R. L. Sinsheimer, *J. Biol. Chem.* **208**, 445 (1954).

[48] W. E. Cohn, D. G. Doherty, and E. Volkin, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.) Vol. II. Johns Hopkins Press, Baltimore, 1952.

[49] E. Volkin and W. E. Cohn, *J. Biol. Chem.* **205**, 767 (1953).

[50] R. B. Merrifield and D. W. Woolley, *J. Biol. Chem.* **197**, 521 (1952).

"standard" material.[46, 47, 50] Since the commercially available resins ha varied over quite a range of divinylbenzene content, it is not possible say, with respect to some reported separations, just what degree of cros linking existed in some of the exchangers used. It is known that the pol nucleotides of the ribonuclease digest, readily separable up to at least tetr nucleotides on 2 % divinylbenzene material in a chloride system,[49] were n separated on 10 % divinylbenzene resin by chloride;[51] very broad peak indicating a poor degree of equilibration and leading to overlapping of components, were obtained (qualitatively similar results have been reported with polypeptides[52]).

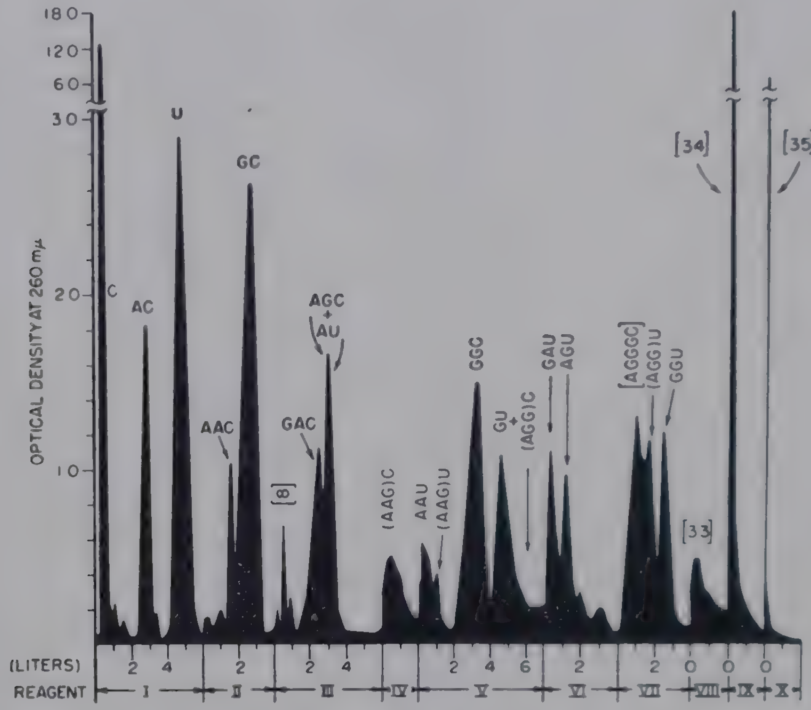

FIG. 18. Separation of the products of ribonuclease digestion of PNA.[49] Exchanger: Dowex-1(2% DVB)-chloride, 400 mesh, 15 cm. × 3.7 cm.². Solution: HCl + NaCl as follows: I, 0.005 N HCl; II, 0.01 N HCl; III–IX, 0.01 N HCl + 0.0125, 0.025, 0.05, 0.1, 0.2, 0.3, and 1 N NaCl, respectively; X, 2 N HCl. Sorbed material: 700 mg. calf liver PNA + 10 mg. ribonuclease in 105 ml. H_2O, 22 hr. at 37°C., + NaOH as required to keep at pH 7.0; pH lowered to 2.0; chilled; centrifuged; supernatant made alkaline with NH_4OH; sorbed.

b. The Ribonuclease Digest

An example of the separation of polynucleotides by anion exchange is shown in Fig. 18.[49] In this separation, the concentration of HCl was held at 0.01 N (after a brief sequence at 0.005 N) with stepwise increments in NaCl to remove the more strongly sorbed polynucleotides. Merrifield and Woolley[50] used stronger HCl rather than NaCl to remove polynucleotides formed by brief acid hydrolysis. Either system (increasing NaCl at constant pH or increasing HCl) can be adapted to gradient development and other anions (e.g., formate) can be used, as has been demonstrated for the acid-soluble polyphosphates.[53]

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[51] C. E. Carter and W. E. Cohn, *J. Am. Chem. Soc.* **72,** 2604 (1950).

[52] S. Moore, personal communication.

It is of some interest to compare the relative positions of the polynucleotides in the elution sequence at pH 2 in a chloride system with those predicted from the positions of the mononucleotides at this pH. Polynucleotides ending in cytidylic acid (e.g., AC and GC) precede those ending in uridylic acid, just as cytidylic acid itself precedes uridylic acid. The nature of the purine nucleotide attached to the end pyrimidine nucleotide exerts an influence predictable from its own behavior, but secondary in importance. Thus, AC precedes GC, AGC precedes GGC, and AAGC precedes AGGC. From the observation that GAC precedes AGC, it would appear that the third nucleotide exerts still less influence than the second one. It should also be noted that the number of phosphate groups exerts the expected influence; thus AC precedes AAC and GU precedes GGU. Finally, it may be remarked that the elimination of the terminal (secondary) phosphoryl group from a dinucleotide makes the residual dinucleoside monophosphate behave like a mononucleotide. Thus, dephosphorylated GC appears in the cytidylic acid region. The behavior of a dephosphorylated trinucleotide has not been investigated.

c. *The Deoxyribonuclease Digest*

The results of Sinsheimer,[47] obtained on the deoxyribonuclease digest of DNA at pH 4–5.5 in chloride and acetate systems, are in general agreement with the above. The order of elution of dinucleotides of cytidylic acid is CC, TC (plus CT), AC, and GC (plus CG). From his earlier studies,[28] it is known that the order of deoxymononucleotides in this pH range is C, T, A, and G. In the trinucleotide region, the order is CCC, CCT, and CTT; these precede GG. In every case where one cytidylic acid residue is replaced by 5-methylcytidylic acid, the polynucleotide is advanced in position (it will be recalled, as in Fig. 11, that 5-methylcytidylic acid precedes cytidylic). Thus MCC precedes CCC, MCT precedes CCT, etc.

IV. Separations Involving Sugar-Borate Complexing

1. SUGARS (BORATE EXCHANGER AND SOLUTION)

Although the sugars have acid ionization properties which could conceivably be used in ion-exchange manipulations, these constants lie so far on the alkaline side (ca. 13) as to be relatively unattractive or unmanageable. However, just as the use of complex-forming acids rendered the strongly sorbed rare earth ions more easily handled, so has it been found that the complexes which sugars form with borate ion possess acidic properties of sufficient strength and of sufficiently differing qualities to permit practical ion-exchange chromatography.

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[53] H. Schmitz, R. B. Hurlbert, and V. R. Potter, *J. Biol. Chem.* **209**, 41 (1954).

Although the neutral sugars ribose and deoxyribose are seldom encountered in the usual nucleic acid digests, there exist other situations in nucleic acid investigations (e.g., biosynthesis and precursor studies) in which it is desirable to have at hand a method for the isolation of one or more sugar components. The separation of a mixture of hexoses and pentoses on a borate-anion exchanger with borate eluting solutions, as originally developed by Khym and Zill,[54, 55] is shown in Fig. 19. Similar separations have been demonstrated for specific groups of monosaccharides,[55] for di-, tri-, and tetrasaccharides,[56] and for sugar alcohols[57] (as well as the uronic acids, which do not require borate for separation[58]).

The dependence of these separations (i.e., of the distribution coefficients) upon the strength of the borate complex and the dependence of this, in turn, upon pH and the structural details of the sugars (e.g., *cis*-glycol groups, furanoid or pyranoid forms, etc.) is discussed by Khym and Zill.[55]

2. Sugar Phosphates (Borate in Solution Only)

a. Nucleosides

This method has been applied to the nucleosides by Jaenicke and von Dahl[59] and by Khym and Cohn[30] (see Fig. 20). It is possible to increase markedly the sorption of the ribonucleosides, whereupon such weakly sorbed nucleosides as cytidine and adenosine become more strongly sorbed and more easily separable. The difference between deoxynucleosides and ribonucleosides with respect to borate complex formation can be exploited to facilitate their separation (see Fig. 21 in comparison to Fig. 4).

b. Sugar Phosphates and the Isomeric Ribose Phosphates

In the usual acid eluting system, the sugar monophosphates behave in an almost identical fashion and are not well separated from one another. The borate complexes, however, have made it possible to separate various hexose phosphates from each other and from ribose-5-phosphate, the latter being strongly affected by borate. The separation of a mixture of sugar

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[54] J. X. Khym and L. P. Zill, *J. Am. Chem. Soc.* **73,** 2399 (1951).

[55] J. X. Khym and L. P. Zill, *J. Am. Chem. Soc.* **74,** 2090 (1952).

[56] G. R. Noggle and L. P. Zill, *Arch. Biochem. and Biophys.* **41,** 21 (1952).

[57] L. P. Zill, J. X. Khym, and G. M. Cheniae, *J. Am. Chem. Soc.* **75,** 1339 (1953).

[58] J. X. Khym and D. G. Doherty, *J. Am. Chem. Soc.* **74,** 3199 (1952).

[59] L. Jaenicke and K. von Dahl, *Naturwissenschaften* **39,** 87 (1952).

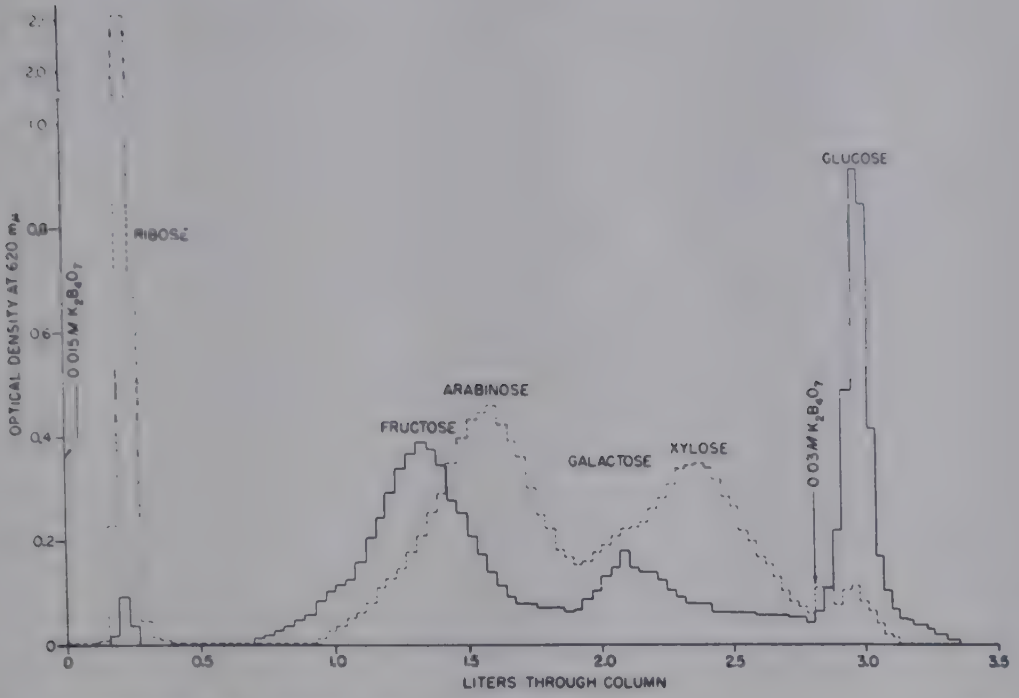

FIG. 19. Separation of sugars by anion exchange in a borate system.[55]
Exchanger: Dowex-1-borate, 11 cm. × 0.84 cm.^{2}.
Solution: tetraborate ($K_2B_4O_7$) as shown at 1 ml./min.
Sorbed material: 2.5 mg. ribose, 5 mg. of other sugars in 10 ml. 0.01 M $K_2B_4O_7$.

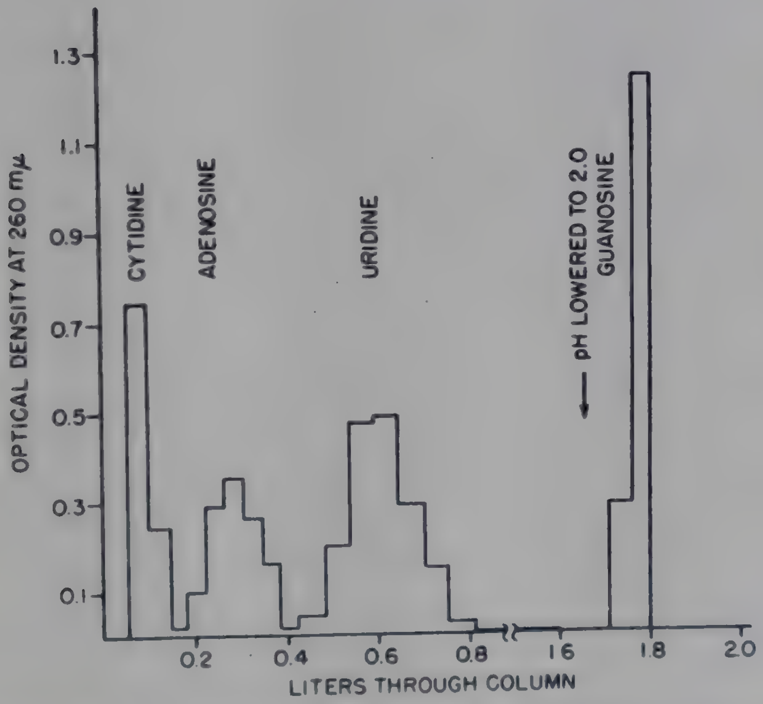

FIG. 20. Separation of ribonucleosides by anion exchange in a chloride system with borate present[30] (see Figs. 3, 4, 5, 21, and 22).
Exchanger: Dowex-1-chloride, 200–400 mesh, 11 cm. × 0.85 cm.^{2}.
Solution: 0.03 M KCl + 0.02 M $K_2B_4O_7$.
Sorbed material: ca. 2 mg. of each nucleoside in 10 ml. 0.01 M $K_2B_4O_7$.

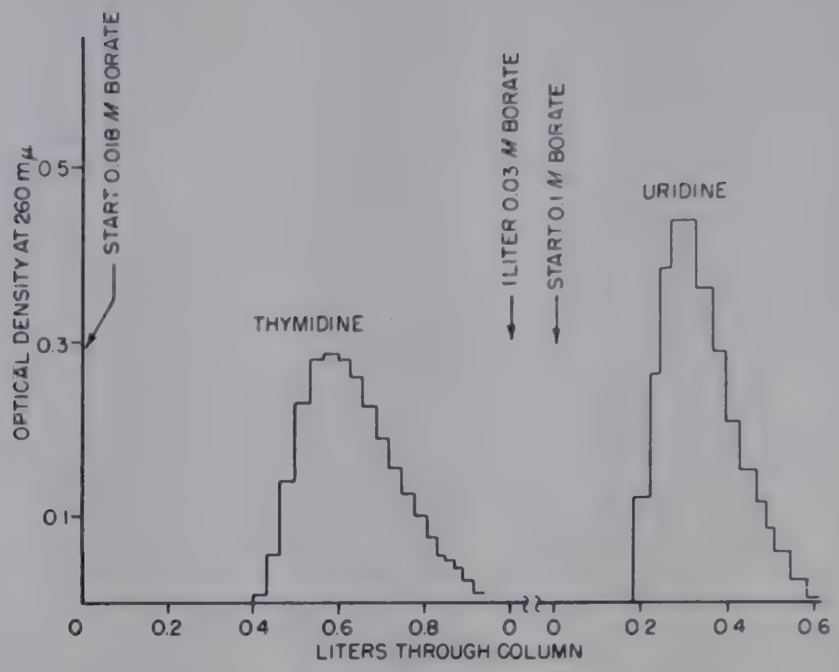

FIG. 21. Separation of uridine and thymidine by anion exchange in a borate system[30] (see Figs. 3, 4, 5, and 20).

Exchanger: same as in Fig. 20 but borate form.

Solutions: $K_2B_4O_7$ as shown, 1 ml./min.

Sorbed material: ca. 3 mg. each of thymidine and uridine in 10 ml. 0.01 M $K_2B_4O_7$

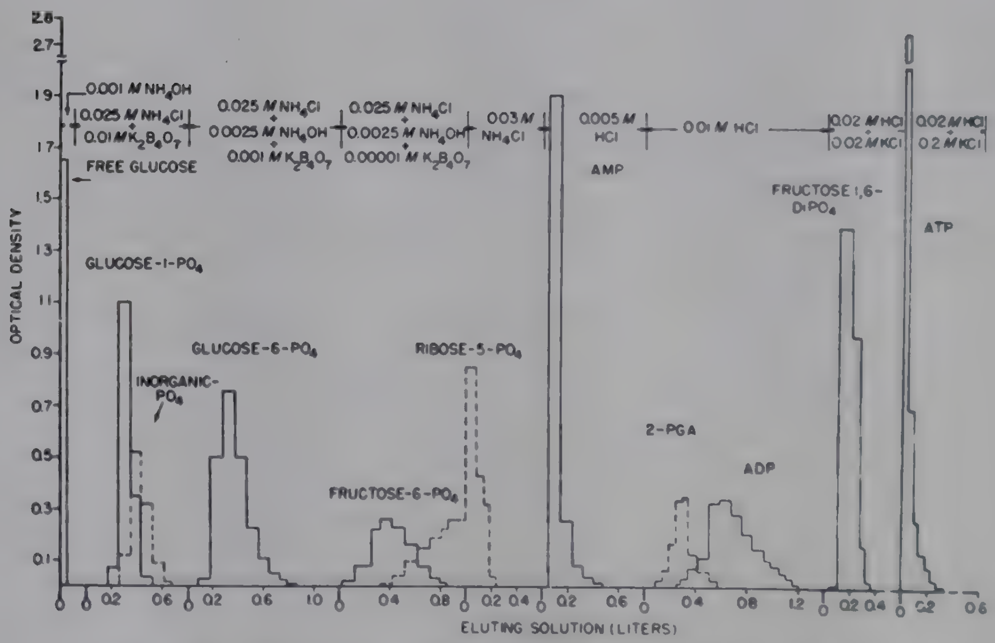

FIG. 22. Separation of sugar acids and sugar phosphates by anion exchange in a chloride system with borate present[60] (see Fig. 20).

Exchanger: Dowex-1-chloride, ca. 300 mesh, 12 cm. × 0.86 cm.².

Solutions: chloride and tetraborate as shown at 3.5 ml./min.

Sorbed material: 2–10 mg. of substance as shown.

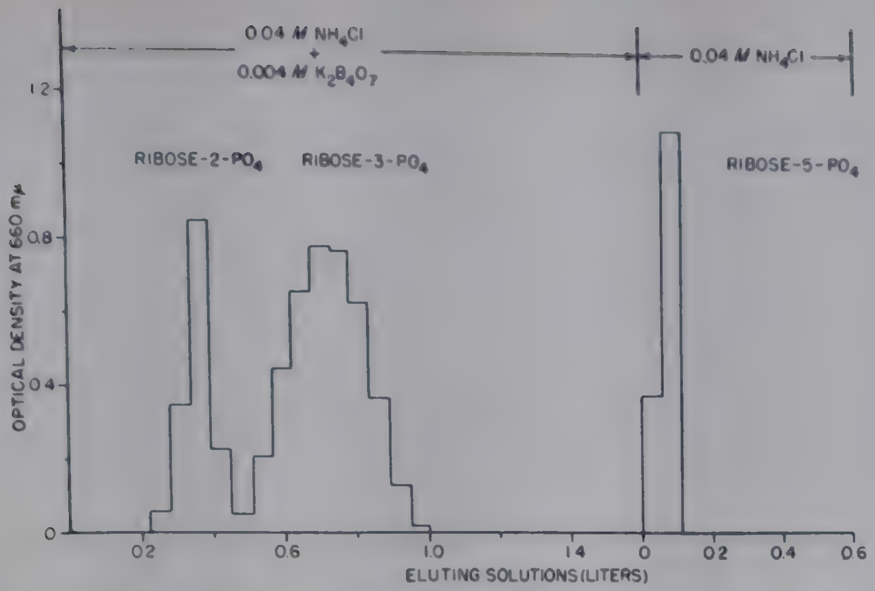

Fig. 23. Separation of isomeric ribose phosphates by anion exchange in a chloride system with borate present[61] (see Figs. 20, 22, and 24).

Exchanger: same as in Fig. 22.

Solutions: chlorides and tetraborate as shown at 2 ml./min.

Sorbed material: 3–8 mg. of each substance shown in 10 ml. of 0.001 *M* NH_4OH.

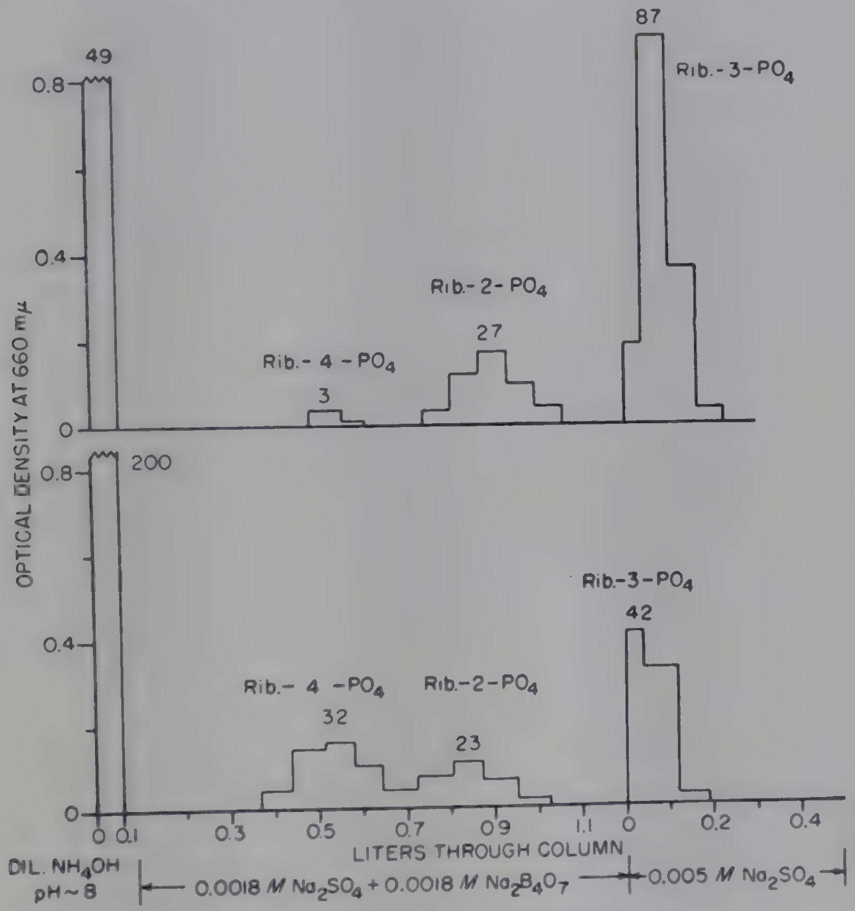

Fig. 24. Separation of isomeric ribose phosphates by anion exchange in a sulfate system with borate present[62] (see Figs. 20, 22, and 23).

Exchanger: same as Figs. 22 and 23, in sulfate form.

Solutions: 0.0018 *M* Na_2SO_4 + 0.0018 *M* $Na_2B_4O_7$ (for ribose-4-phosphate and ribose-2-phosphate), 0.005 *M* Na_2SO_4 (for ribose-3-phosphate).

Sorbed material: (a) 10 mg. commercial adenylic acid, 1 g. Dowex-50-H, 1 ml. H_2O; heated at 100°C. for 20 min., with stirring; filtered; NH_4OH added to supernatant; sorbed; (b) same as (a), but doubled quantities and heated for 2 hr.

phosphates and related substances, utilizing borate complexing, is shown in Fig. 22.[60] The separation of ribose-2- and-3-phosphates, which was an essential part of the preparation and characterization of these two substances and of their identification as parts of the adenylic and guanylic acids *a* and *b*, respectively, is shown in Fig. 23.[61, 62]

In the course of these separations, it was found that an unexpected isomer of ribose phosphate appeared[62, 63] whenever ribose-2- or 3-phosphate

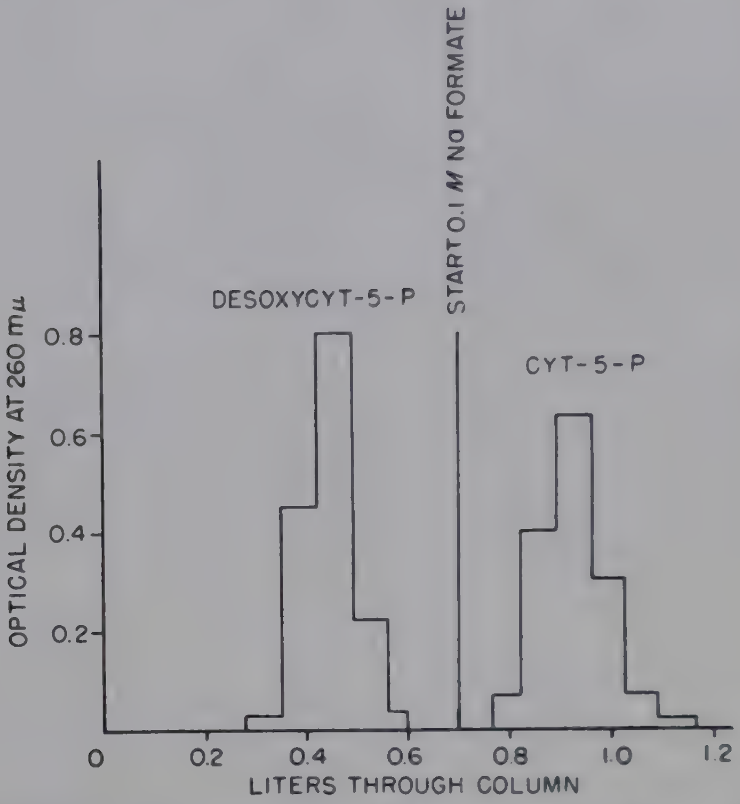

FIG. 25. Separation of deoxycytidine-5′-phosphate and cytidine-5′-phosphate by anion exchange in a formate system with borate present.[64]

Exchanger: same as Figs. 22, 23, and 24, in formate form.

Solutions: 0.1 *M* Na formate plus .0005 *M* tetraborate (peak 1); 0.1 *M* Na formate (peak 2).

Sorbed material: ca. 5 mg. each in 10 ml. 0.01 *M* NH_4OH.

was heated in acid for a period which produced a large degree of hydrolysis to free ribose (which was identified as such by the method of Khym and Zill[54]). This new isomer, which was separable in a sulfate (also in a chloride) system without borate[63] from the 2 and 3 isomers and partially separated from the 5, has been identified as the 4 isomer. In Fig. 24[62] are shown the appearance of ribose-4-phosphate upon acid treatment and its separation from ribose-2- and 3-phosphates in a sulfate-plus-borate system (the borate influences the position of the 3-phosphate only, as neither the 4- nor the 2- derivatives form complexes appreciably with borate).[63]

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[60] J. X. Khym and W. E. Cohn, *J. Am. Chem. Soc.* **75,** 1153 (1953).

[61] J. X. Khym, D. G. Doherty, E. Volkin, and W. E. Cohn, *J. Am. Chem. Soc.* **75,** 1262 (1953).

[62] J. X. Khym and W. E. Cohn, *J. Am. Chem. Soc.* **76,** 1818 (1954).

[63] J. X. Khym, D. G. Doherty, and W. E. Cohn, for *J. Am. Chem. Soc.*, **77,** in press.

[64] J. X. Khym and W. E. Cohn, *Biochim. et Biophys. Acta* **15,** 139 (1954).

Ribose-1-phosphate, in sulfate-borate systems, precedes only very slightly ribose-3-phosphate.[63] In the absence of borate, it would be expected to precede ribose-4-phosphate. Its acid-lability precludes its presence in the acid hydrolysates used to produce the other four isomers, but we may list the order of elution, in the sulfate-borate systems, as 4, 2, 1, 3, 5.[63]

c. *Nucleotides*

An extension of this method to the separation of cytidine-5′-phosphate and deoxycytidine-5′-phosphate yields the expected result: the latter is not complexed and thus precedes the ribose derivative (Fig. 25). This should be applicable to all such pairs.

V. RELATED REVIEWS

The ion-exchange separation of the products of alkaline, diesterase and nuclease digests of PNA and DNA, together with the methods of preparing these digests, is described in a parallel review[65] that includes the spectrophotometric constants which are so useful in following the course of separation. Detailed directions for the ion-exchange separation and concentration of the PNA and DNA mononucleotides are given elsewhere.[66]

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[65] W. E. Cohn, *in* Colowick and Kaplan, Methods in Enzymology, Vol. II, Academic Press, New York, in press.

[66] W. E. Cohn and J. X. Khym, also W. E. Cohn, E. Volkin and J. X. Khym, *in* W. W. Westerfeld, Biochemical Preparations, Vol. IV, John Wiley & Sons, New York, in press.

rated, the thick papers Whatman No. 3 (grained surface) and No. 3MM (smooth surface) are useful. No. 3 is approximately 2.2 times as thick as No. 1, and more than 10 mg. of a substance can be separated from a mixture applied as a band across a single sheet (18¼" x 22½"). 5-Hydroxymethylcytosine was isolated from bacteriophage DNA on this type of chromatogram in amounts adequate for crystallization and analysis.[11]

Filter papers contain a certain amount of ultraviolet-absorbing material which may be eluted by a chromatographic solvent, especially if the latter is acid, and collect in a band at or behind the solvent front. This can be removed by prior washing of the paper, which is desirable in preparative work. In quantitative work, it may generally be allowed for by taking appropriate blanks. For successful chromatography of phosphoric esters in many solvents, however, thorough washing of the paper is essential, as otherwise the presence of metallic ions may cause streaking or double spots.[12, 13]

With a given solvent system, precise R_F values[14] are influenced by (a) the composition of the vapor phase in the chromatography vessel, which, ideally, should be in equilibrium with the solvent mixture before a run is started, (b) the temperature, which affects partition coefficients, (c) the direction (ascending or descending) and length of run, since the composition of the solvent may change during its passage through the paper,[15] and (d) the paper, of which some variation is found even between batches of one grade. If these conditions are adequately controlled R_F values can be accurately reproduced.[16] However, as has frequently been observed, a characteristic pattern of the spots can be maintained despite considerable variation in absolute values of R_F, so that precise control is usually unnecessary. The sensitivity to environmental conditions depends on the particular solvent system.

III. Detection of Purine and Pyrimidine Derivatives on Filter Paper

1. PURINE AND PYRIMIDINE BASES

In the earlier experiments, a variety of means were tried for determining the positions of nucleic acid derivatives on filter paper. Hotchkiss[2] cut the paper into narrow bands, each of which was eluted for measurement of its ultraviolet extinction in the spectrophotometer. Vischer and Chargaff[1, 17] treated the paper with salts of mercury, and, after the excess had been washed out, the mercury fixed by the purine and pyrimidine bases was made visible by conversion to black mercuric sulfide. Another chemical method of detecting purines[18] depends on their conversion to silver sal Purines may also be made visible by staining their mercury complexes wi eosin or bromphenol blue, or by their fluorescence after exposure to chl ine.[19] A microbiological method for detecting purines and pyrimidine nucle sides on paper chromatograms with the aid of deficient strains of *Ophio toma* has been used by Fries *et al.*[20] In general, however, the most convenie techniques are those taking advantage of the absorption of ultraviolet lig by the nucleic acid bases.

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[11] G. R. Wyatt and S. S. Cohen, *Biochem. J.*, **55,** 774 (1953).

[12] C. S. Hanes and F. A. Isherwood, *Nature* **164,** 1107 (1949).

[13] K. C. Smith and F. W. Allen, *Federation Proc.* **12,** 269 (1953).

[14] Defined[7] as $R_F = \dfrac{\text{movement of band}}{\text{movement of advancing front of liquid}}$

[15] L. Horner, W. Emrich, and A. Kirshner, *Z. Elektrochem.* **56,** 987 (1952).

[16] E. C. Bate-Smith, *Biochem. Soc. Symposia* (*Cambridge, Engl.*) **No. 3,** 62 (1949).

[17] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176,** 703 (1948).

When a paper chromatogram is examined under an ultraviolet lamp ha ing a high emission in the range of maximal nucleic acid absorption, an with visible light efficiently filtered out, spots of nucleic acid component appear as dark regions against the background fluorescence of the filte paper.[21-23] A low-pressure mercury resonance lamp is suitable, and it is re ported that 0.2 μg. of adenine spread over a circle 1.5 cm. in diameter ca be detected.[24]

Another procedure of approximately equal sensitivity, which is more laboriou but provides a permanent record of each chromatogram, consists in making photo graphic contact prints in ultraviolet light.[25, 26] A medium- or high-pressure mercur lamp is used, with a filter system which isolates the 253.7-mμ and 265-mμ emissio lines.[27] The dried paper chromatogram is pinned over a sheet of photographic pape (a contact document or photostat paper is suitable) on a board and exposed to the lamp for an appropriate time (usually less than a minute). In the developed print, the positions of substances absorbing ultraviolet light of the wavelength used appear as white areas on a dark background. These spots may then be traced on the chromatogram itself. Slightly greater sensitivity is claimed for a technique identical in principle but utilizing the 257-mμ and 275-mμ emission lines from a cadmium arc.[28]

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[18] R. M. Reguera and I. Asimov, *J. Am. Chem. Soc.* **72,** 5781 (1950).

[19] H. Michl, *Naturwissenschaften* **40,** 390 (1953).

[20] N. Fries and U. Bjorkman, *Physiol. Plantarum* **2,** 212 (1949); N. Fries and B. Forsman, *ibid.* **4,** 410 (1951).

[21] E. R. Holiday and E. A. Johnson, *Nature,* **163,** 216 (1949).

[21a] E. Chargaff, B. Magasanik, R. Doniger, and E. Vischer, *J. Am. Chem. Soc.* **71,** 1513 (1949).

[22] C. E. Carter, *J. Am. Chem. Soc.* **72,** 1466 (1950).

[23] T. Wieland and L. Bauer, *Angew. Chem.* **63,** 511 (1951).

[24] Marshak[84] reports this sensitivity using a General Electric Co. lamp No. G8T5 equipped with Corning filter No. 9863. "Mineralight" lamps have been widely used, but are rather less sensitive. The effect has been photographed by J. P. Goeller and S. Sherry, *Proc. Soc. Exptl. Biol. Med.* **74,** 381 (1950).

[25] R. Markham and J. D. Smith, *Nature* **163,** 250 (1949).

[26] R. Markham and J. D. Smith, *Biochem. J.* **45,** 294 (1949).

[27] Markham and Smith[50] recommend the Mazda MB/V lamp with the glass bulb removed, and a filter system made with two 25-ml. fused silica round-bottomed flasks containing, respectively, a solution of cobalt and nickel sulfates ($CoSO_4 \cdot 7H_2O$ 10 g. and $NiSO_4$ 35 g. per 100 ml.) and dry chlorine gas. The writer has found satisfactory a General Electric AH-4 lamp equipped with this filter system. The chlorine gas filter may be replaced by a 1-cm. layer of chlorine dissolved in carbon tetrachloride.[39] The system transmits too much visible light to be satisfactory for making spots visible by fluorescence quenching, but by adding a Corning filter No. 9863 or by viewing the chromatograms through a blue filter, one may use it in this way too.

It has been pointed out by Smith and Markham[29] that guanine and compounds containing it fluoresce quite strongly in light of wavelengths 253.7 and 265 mμ, and are thus easily differentiated from other nucleic acid derivatives. Acid conditions are required, and may be created by exposing the chromatogram to fumes of hydrochloric acid. Xanthine behaves similarly.[29a] The effect may be recorded photographically by inserting between the chromatogram and the photographic paper a sheet of cellulose nitrate, which transmits only the fluorescent light. 8-Azaguanine and its compounds fluoresce under both acid and basic conditions,[29a] and may be detected on chromatograms by their fluorescence with greater sensitivity than by their absorption of ultraviolet light.[30]

With the device described by Paladini and Leloir,[31] in conjunction with the Beckman spectrophotometer, a continuous record may be obtained of the ultraviolet absorption of strip chromatograms.

2. Nucleosides and Nucleotides

The techniques using ultraviolet light described above are of course also applicable to nucleosides and nucleotides. Some color reactions of the sugar and phosphate portions of these compounds are also useful on occasion, as in identification of unknowns or where it is necessary to use a chromatographic solvent which itself absorbs in the ultraviolet range.

Buchanan, Dekker, and Long[32] have developed means of detecting both ribo- and deoxyribonucleosides on chromatograms by reactions of the sugars. The *cis*-glycol structure present in ribosides may be oxidized either with periodate, the resulting aldehydes being made visible with Schiff's reagent, or with lead tetraacetate, in which case white spots remain when the uncombined lead on the paper is converted to lead dioxide. Other substances having this configuration, including adenosine-5′-phosphate, react, but riboside-2′- and -3′-phosphates and deoxyribosides do not. The sensitivity of both methods is reported as about 20 μg. of nucleoside. Deoxyribosides on paper can be detected by adaptations of the Dische diphenylamine reaction or of the Feulgen reaction, or of the reaction with cysteine.[33] The last method is sensitive to 10 μg. of deoxyriboside. [Cf. Chapters 9 and 17.]

The positions of deoxyribosides on paper chromatograms have also been determined by virtue of their ability to promote growth of *Labctobacillus leichmannii*.[34]

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[28] J.-E. Edström, *Nature* **168,** 876 (1951).

[29] J. D. Smith and R. Markham, *Biochem. J.* **46,** 509 (1950).

[29a] J. Kream and E. Chargaff, *J. Am. Chem. Soc.* **74,** 4274 (1952).

[30] R. E. F. Matthews, *Nature* **171,** 1065 (1953).

[31] A. C. Paladini and L. F. Leloir, *Anal. Chem.* **24,** 1024 (1952).

[32] J. G. Buchanan, C. A. Dekker, and A. G. Long, *J. Chem. Soc.* **1950,** 3162.

[33] J. G. Buchanan, *Nature* **168,** 1091 (1951).

[34] V. Kocher, R. Karrer, and H. R. Müller, *Intern. Z. Vitaminforsch.* **21,** 403 (1950).

Nucleotides and other phosphoric acid esters on paper chromatograms can be detected by spraying with an acid molybdate solution, partial hydrolysis of the ester and reduction of the resulting phosphomolybdate complex to a blue-colored compound.[35] The necessary hydrolysis may be effected by heating the papers after spraying, by ultraviolet irradiation,[36] or by previous spraying with a solution of phosphatase.[37, 38] It is claimed that 0.1 μg. P can be detected. If the water in the reagent is partially replaced by acetone[39] the papers may be dipped in it instead of sprayed Nucleotides can also be detected by fixation of uranium,[40] and phosphates, by fixation of ferric iron;[41] these reactions avoid the need for hydrolysis.

IV. Solvent Systems

1. General and Theoretical Considerations

The solvent systems with which successful separations were first obtained on paper chromatograms consisted of organic fluids saturated with water. Their effect was satisfactorily interpreted as resulting from the partition of solutes between a water-poor mobile phase and a water-rich phase held by the strongly hydrophilic cellulose fibers; and for a number of amino acids and carboxylic acids, partition coefficients calculated from R_F values on the basis of this theory agree well with the coefficients directly measured.[7, 42] A minor role may be played by adsorption and ion exchange, since the cellulose fibers are electronegative in water, and carry a small number of aldehyde and carboxyl groups.[5] It was subsequently found that the solvent need not be saturated with water, since the binding of water by the cellulose results in a partition effect with miscible solvents just as with water-saturated ones.[43] In a further innovation, it was discovered that separations may be obtained in the absence of any organic solvent, using salt solutions, or even with water alone. Separations with water as the solvent, apparently due to adsorption by the paper, may also be interpreted as the result of partition between water and a water–cellulose complex. [See below, Section IV.2.]

The influence of the composition of the solvent system on the movement of substances has been discussed in terms of partition theory by Martin.[3, 44] The following factors are among the most important which may be utilized in preparing solvents to effect desired separations and in using paper chromatography in the identification of unknown substances.

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[35] Hanes and Isherwood[12] spray the chromatograms at a rate of 1 ml. per 100 cm.^{2} with a solution containing: 5 ml. 60% $HClO_4$, 10 ml. *N* HCl, 25 ml. 4% $(NH_4)_2MoO_4$, and water to 100 ml.; then heat them to 85° for 7 min., and subsequently expose them to H_2S. Benson *et al.*[42] describe a similar reagent.

[36] R. S. Bandurski and B. Axelrod, *J. Biol. Chem.* **193,** 405 (1951).

[37] N. G. Doman and Z. S. Kagan, *Biokhimiya* **17,** 719 (1952), seen only in abstract, *Chem. Abstracts* **47,** 4795 (1953).

[38] E. Fletcher and F. H. Malpress, *Nature* **171,** 838 (1953).

[39] S. Burrows, F. S. M. Grylls, and J. S. Harrison, *Nature* **170,** 800 (1952).

[40] B. Magasanik, E. Vischer, R. Doniger, D. Elson, and E. Chargaff, *J. Biol. Chem.* **186,** 37 (1950).

[41] H. E. Wade and D. M. Morgan, *Nature* **171,** 529 (1953)

[42] A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka, *J. Am. Chem. Soc.* **72,** 1710 (1950).

[43] H. R. Bentley and J. K. Whitehead, *Biochem. J.* **46,** 341 (1950).

a. Water content

By using a miscible organic solvent, the water content may be varied over a wide range. As water is added to the moving phase, the rates of migration of solutes will increase in proportion to their polarity. For example, by altering the water content of propanol–water mixtures, the relative positions of adenine and adenylic acid[26] or 5-methylcytosine and 5-hydroxymethylcytosine[45] may be reversed.

b. pH

Since ionization will alter the partition of a solute in favor of the aqueous phase, one can regulate the relative rates of movement of ionizable substances by control of pH, having regard to their dissociation constants. [Cf. *Cohn*, Chapter 6, and *Jordan*, Chapter 13.] Thus, in neutral aqueous *n*-butanol uracil migrates more rapidly than cytosine, but if the solvent is made basic by the presence of sufficient ammonia the movement of uracil and thymine may be slowed until the former has an R_F less than that of cytosine. This may be explained by ionization of enolic hydroxyls ($pK_1 =$ 9.5 and 9.9 for uracil and thymine, respectively[46]), the hydroxyl of cytosine having too high a pK (12.2) to be more than slightly affected by ammonia. The relative mobilities are of course sensitive to the precise concentration of ammonia (cf. Table I, solvents *a*, *b*, and *c*). Addition of strong acid or base to a chromatographic solvent may have further effects on partition coefficients; for example, hydrochloric acid added to aqueous alcohols decreases the R_F's of all the purine and pyrimidine bases, but the effect is differential and at about 1 *N* HCl the relative positions of adenine and cytosine are interchanged.[47]

c. Nature of the Organic Components

The importance of van der Waals' forces and of hydrogen bonding in determining the partition of a system has been stressed by Martin.[44] When solvent and solute are similar in structure, for example both aromatic or both aliphatic, closer fit between their molecules, resulting in greater van der Waals' forces and higher R_F values, may be expected. This principle is of limited utility in separating nucleic acid bases, where we are concerned exclusively with two similar ring systems, but does account for such facts the better separation of 5-methylcytosine from cytosine with increasin chain length of the alcohol used.[48] The nucleic acid derivatives have num erous possibilities for hydrogen bonding, which undoubtedly contribut to the different order of movement of the nucleotides given by solvent active in forming hydrogen bonds, such as phenol and butyric acid, tha by aliphatic alcohols.

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[44] A. J. P. Martin, *Biochem. Soc. Symposia* (*Cambridge, Engl.*), **No. 3,** 4 (1949).
[45] G. R. Wyatt and S. S. Cohen, *Nature* **170,** 1072 (1952).
[46] D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta* **9,** 199 (1952).
[47] G. R. Wyatt, *Biochem. J.* **48,** 584 (1951).

d. Salt Content

Salt will generally decrease the mutual solubility of water and an organi solvent, and will thus alter the partition of a chromatographic solvent sys tem, and for this reason the local presence of salt may cause distorted spots Addition of salt to a system selectively slows the movement of solutes, and relatively strong salt solutions containing little or no organic solvent may be used to obtain chromatographic separations on filter paper. Hagdahl and Tiselius[49] have separated amino acids using 3 M phosphate buffer as the solvent; they term this "salting-out chromatography" and attribute the effect to reductions in solubility due to the presence of salt, with consequent increase in apparent adsorption. With similar "salting-out solvents" (5 % ammonium citrate or sodium or potassium phosphate overlayered with isoamyl alcohol, which is slightly soluble in water and has the effect of producing more compact spots), Carter[22] has obtained separations of nucleic acid components. The principle has been further applied by Markham and Smith,[50] who used 0.8 saturated ammonium sulfate containing 2 % isopropanol. It is notable that these systems separate substances in a different order from the more usual organic systems, and that they are capable of resolving nucleoside-2′- and -3′-phosphates, a separation not accomplished in any system of the more usual type.

Two practical considerations to be borne in mind when preparing solvent systems are: (a) it is preferable to select volatile substances (ammonium sulfate might with advantage be replaced by ammonium carbonate) and (b) when working with nucleic acid derivatives it is of course particularly desirable to avoid substances absorbing in the ultraviolet range.

2. Separation of Purine and Pyrimidine Bases and Nucleosides

At the time of writing, over eighty solvent systems have been described for separation of nucleic acid components on paper chromatograms. Fortunately for the reviewer and for those using this technique, relatively few of these show real advantages over others, and some of the simplest mixtures remain the most valuable. In Table I are listed the reported R_F values of some purine and pyrimidine bases and nucleosides in a number of solvent systems selected to include (a) those which, in the writer's estimation, have proved the most broadly useful, and (b) representatives of different types of mixture which have been tried with some degree of success. It is obvious that for some purposes other solvents will be preferable to those listed. For completeness, the R_F values reported by the original authors have been supplemented with some determined by the writer.

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[48] G. R. Wyatt, *Biochem. J.* **48,** 581 (1951).

[49] L. Hagdahl and A. Tiselius, *Nature* **170,** 799 (1952).

[50] R. Markham and J. D. Smith, *Biochem. J.* **49,** 401 (1951).

Aqueous *n*-butanol, with and without added ammonia, was one of the first solvents tested for separation of nucleic acid derivatives[2, 17] and remains among the most useful (Table I, solvents *a*, *b*, and *c*). Butanol saturated with water has usually been used; however, the solution is most conveniently made up at a fixed percentage composition slightly under-saturated, thus making its composition independent of temperature.[51] Since R_F values are rather low, it is advantageous to use the descending method and to cut the end of the paper to a number of teeth, from which the solvent is allowed to drip.[26] Ammonia may be added either to the solvent irrigating the paper or to that in the bottom of the tank; owing to the volatility of ammonia, the latter practice affords the more constant conditions. It has the effect of slowing the movement of substances with acidic substituents, and the results given by two different concentrations are illustrated in Table I (solvents *b* and *c*). With butanol–water–ammonia, all of the purine and pyrimidine bases known to occur in nucleic acids (except 5-hydroxymethylcytosine, which runs close to guanine) may be resolved from one another. In addition to the authors cited in the Table, Chargaff, *et al.*[52, 52a] Marshak and Vogel,[53] and others have used butanol–ammonia mixtures for quantitative separation of nucleic acid components.

Addition of formic acid to aqueous butanol (solvent *d*) results in more rapid movement of acidic substances such as uracil, thymine, xanthine, and hypoxanthine.

If butanol is saturated with a saturated solution of boric acid instead of with water, ribosides, by virtue of their *cis*-diol configuration, form borate complexes and do not move. Complete separation of free bases from ribosides may thus be obtained.[54]

Admixture of various other substances with butanol–water systems has been tried, generally without much advantage. Butanol may be saturated with a 10% solution of urea, instead of water, with similar results.[22] Mixing morpholine or diethylene

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[51] *n*-Butanol satd. with water at 20° contains 84% by vol. of butanol, calcd. from data given by A. Seidell, "Solubilities of Organic Compounds," Vol. 2, p. 266. Van Nostrand Co., New York, 1941.

[52] E. Chargaff, R. Lipshitz, C. Green, and M. E. Hodes, *J. Biol. Chem.* **192,** 223 (1951).

[52a] C. Tamm, H. S. Shapiro, R. Lipshitz, and E. Chargaff, *J. Biol. Chem.* **203,** 673 (1953).

[53] A. Marshak and H. J. Vogel, *J. Biol. Chem.* **189,** 597 (1951).

[54] I. A. Rose and B. S. Schweigert, *J. Am. Chem. Soc.* **73,** 5903 (1951).

TABLE I

R_F VALUES OF PURINE AND PYRIMIDINE BASES AND NUCLEOSIDES

	Solvent									
	n-Butanol[a]	*n*-Butanol–NH_3[b]	*n*-Butanol–NH_3[c]	*n*-Butanol–formic acid[d]	Isopropanol–NH_3[e]	Isopropanol–HCl[f]	Collidine–quinoline[g]	Isobutyric acid–NH_3[h]	Na_2HPO_4–isoamyl alc.[i]	Water (pH
Adenine	0.38	0.28	0.40	0.33	0.37	0.32	0.34	0.83	0.44	0.
Guanine	0.15	0.11	0.15	0.13	0.16	0.22	0.22	0.70	0.02	0.4
Hypoxanthine	0.26	0.12	0.19	0.30	0.16	0.29	0.44	0.69	0.57	0.6
Xanthine	0.18	0.05	0.01	0.24	0.11	0.21	0.62	0.60	0.49	0.6
Uracil	0.31	0.19	0.33	0.39	0.38	0.66	0.74	0.67	0.73	0.7
Thymine	0.52	0.35	0.50	0.56	0.52	0.76	0.84	0.78	0.73	0.7
Cytosine	0.22	0.24	0.28	0.26	0.32	0.44	0.21	0.80	0.73	0.7
5-Methylcytosine	0.29	0.27	0.36	—	0.37	0.52	—	—	—	0.7
5-Hydroxymethylcytosine	0.13	0.12	—	—	0.25	0.44	—	—	—	0.7
Adenosine	0.20	0.22	0.33	0.12	0.31	0.34	—	0.91	0.54	0.4
Guanosine	0.15	0.03	0.10	0.17	0.13	0.30	—	0.59	0.62	0.6
Inosine	—	0.03	0.08	—	0.14	0.30	—	—	—	0.8
Uridine	0.17	0.08	—	0.25	0.31	0.64	—	0.60	0.79	0.8
Cytidine	0.12	0.11	0.15	0.18	0.28	0.45	—	0.73	0.76	0.76
Adenine DR[k]	0.35	—	0.41	—	—	—	—	0.91	0.55	0.47
Guanine DR	0.21	—	0.18	—	—	—	—	0.67	0.62	—
Hypoxanthine DR	0.23	—	0.17	—	—	—	—	0.70	0.70	0.80
Uracil DR	0.38	—	0.34	—	—	—	—	0.67	0.79	0.83
Thymine DR	0.51	0.40	0.48	—	0.57	0.81	—	0.75	0.78	0.77
Cytosine DR	0.23	—	0.26	—	—	0.60	—	0.83	0.77	0.75
5-Methylcytosine DR	0.25	—	—	—	—	—	—	—	0.76	—

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[a] 86% (vol./vol.) aq. *n*-butanol; Whatman No. 1 paper, descending; Markham and Smith, *Biochem. J* **45,** 294 (1949). Values for deoxyribosides are from Buchanan,[33] using *n*-butanol satd. with water; values for methylcytosine and hydroxymethylcytosine detd. by the reviewer.

[b] 86% (vol./vol.) aq. *n*-butanol, with 5% by vol. of concd. NH_3 soln. (sp. gr. 0.880) added to solvent in bottom of tank; Whatman No. 1, descending; Markham and Smith, *op. cit.* Values for methylcytosine, hydroxymethylcytosine, inosine, and thymine deoxyriboside detd. by the reviewer.

[c] *n*-Butanol satd. with water at about 23° 100 ml., 15 *N* NH_4OH 1 ml.; Whatman No. 4, ascending; MacNutt, *Biochem. J.* **50,** 384 (1952). Values for xanthine, adenosine, and guanosine are from Hotchkiss, *J. Biol Chem.* **175,** 315 (1948), with a similar solvent system.

[d] *n*-Butanol 77%, water 13%, formic acid 10% by vol.; Whatman No. 1, descending; Markham and Smith, *op. cit.*

[e] Isopropanol 85 ml., water 15 ml., concd. (28%) NH_3 soln. 1.3 ml.; Whatman No. 1, descending; Hershey *et al.*, *J. Gen. Physiol.* **36,** 777 (1953). R_F values at 20–23° detd. by the reviewer.

[f] Isopropanol 170 ml., concd. HCl (sp. gr. 1.19) 41 ml., water to make 250 ml.; Whatman No. 1, descending; Wyatt, *Biochem. J.* **48,** 584 (1951). Values redetd. at 20–23° by the reviewer.

[g] Collidine 1 vol., quinoline 2 vol., mixt. satd. with 1.5 vol. water; Schleicher and Schüll No. 597 paper, descending, at about 22°; Vischer and Chargaff, *J. Biol. Chem.* **176,** 703 (1948).

[h] Isobutyric acid 400 ml., water 208 ml., 25% NH_3 soln. 0.4 ml.; Whatman No. 4, descending, at 22°; Löfgren, *Acta Chem. Scand.* **6,** 1030 (1952); excepting deoxyribosides, for which solvent and conditions are as in footnote *e*, Table II, and R_F values are calcd. from the relative mobilities given by Tamm *et al.*, *J. Biol. Chem.* **203,** 6[illegible]3 (1953), taking the R_F of thymidine as 0.75.

[i] 5% aq. Na_2HPO_4 satd. with isoamyl alc., both aq. and nonaq. phases being present in the trough; Whatman No. 1, descending; Carter, *J. Am. Chem. Soc.* **72,** 1466 (1950). Values for deoxyribosides are from Buchanan, *Nature* **168,** 1091 (1951).

[j] Water adjusted to pH 10 with *N* NH_4OH; Whatman No. 1, ascending, 22–23°; Levenbook, personal communication, 1953. Values for methylcytosine and hydroxymethylcytosine detd. by the reviewer.

[k] DR = deoxyriboside.

glycol with butanol permits addition of more water and gives higher R_F values, without markedly altering the order of separation of the bases.[17] Addition of ethanol gives higher R_F values with some loss of resolution.[26] Dioxane[28] and 2-methoxyethanol (methyl cellosolve)[55] have been added to butanol–water mixtures, and also lead to increased R_F values. Water-saturated *n*-butanol has been mixed with acetic acid, with ethyl acetate and morpholine, and with methyl glycol and morpholine with some success in separating deoxyribosides.[56]

Amyl alcohol saturated with water gives inconveniently low R_F values for most substances.[26] Isopropanol–water–ammonia (Table I, solvent *e*) has been found a useful mixture by Hershey, *et al.*:[57] it resolves 5-hydroxymethylcytosine from the other bases, but adenine and uracil run together, and the bases are less well separated from their ribosides than in butanol. Mixtures of tetrahydrofurfuryl alcohol with propanol and amyl alcohol buffered at different pH's have also been tried,[58] with some success in separation of nucleotides, but for separation of the bases and nucleosides they appear to be inferior to simple butanol solvents. Several other mixtures of alcohols with NH_3 and HCl have also been tested on a limited range of substances,[59] and the R_F values of orotic acid[60] and of uric acid and its riboside[61] in several solvents have been recorded.

Solvent systems based on collidine and quinoline instead of alcohols have been tested by Vischer and Chargaff.[17] In these (e.g., Table I, solvent *g*) the bases migrate in a different order, xanthine and hypoxanthine running more rapidly and cytosine much more slowly. However, the strong absorption of ultraviolet light by these solvents is a serious drawback.

Isobutyric acid mixed with water and ammonia, as tested by Löfgren,[62] distributes the bases and nucleosides in a different order from other solvents, as shown in Table I (solvent *h*), although R_F values are grouped undesirably close together. (Compare also Tamm *et al.*[52a]) The effect is partially retained, with better spread of R_F values, in a solvent containing *n*-butanol (75 ml.), isobutyric acid (37.5 ml.), water (25 ml.), and ammonia (2.5 ml. of 25% soln.). A mixture containing piperidine, tried by the same author, gave rather poor separations.

A limitation of all these neutral or weakly basic or acidic solvent systems for quantitative analysis of nucleic acids is their low capacity for guanine. Because of its insolubility, guanine in amounts of more than a few micrograms tends to form "tails" or double spots, or to remain partly at the origin. This difficulty may be avoided by use of solvents containing relatively high concentrations of hydrochloric acid. Such a system was first used by Smith and Markham[29] for separation of the purine bases and pyrimidine nucleotides obtained by mild acid hydrolysis of PNA (Table II, solvent *a*). A mixture containing isopropanol and hydrochloric acid (Table I, solvent *f*) was subsequently developed for separation of the bases from DNA.[47]

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[55] S. G. Laland, W. G. Overend, and M. Webb, *J. Chem. Soc.* **1952**, 3224.
[56] W. S. MacNutt, *Biochem. J.* **50**, 384 (1952).
[57] A. D. Hershey, J. Dixon, and M. Chase, *J. Gen. Physiol.* **36**, 777 (1953).
[58] D. C. Carpenter, *Anal. Chem.* **24**, 1203 (1952).
[59] B. Bheemeswar and M. Sreenivasaya, *Current Sci.* (*India*) **20**, 61 (1951).
[60] E. Leone and E. Scala, *Boll. soc. ital. biol. sper.* **26**, 1223 (1950).
[61] E. Leone and D. Guerritore, *Boll. soc. ital. biol. sper.* **26**, 609 (1950).
[62] N. Löfgren, *Acta Chem. Scand.* **6**, 1030 (1952).

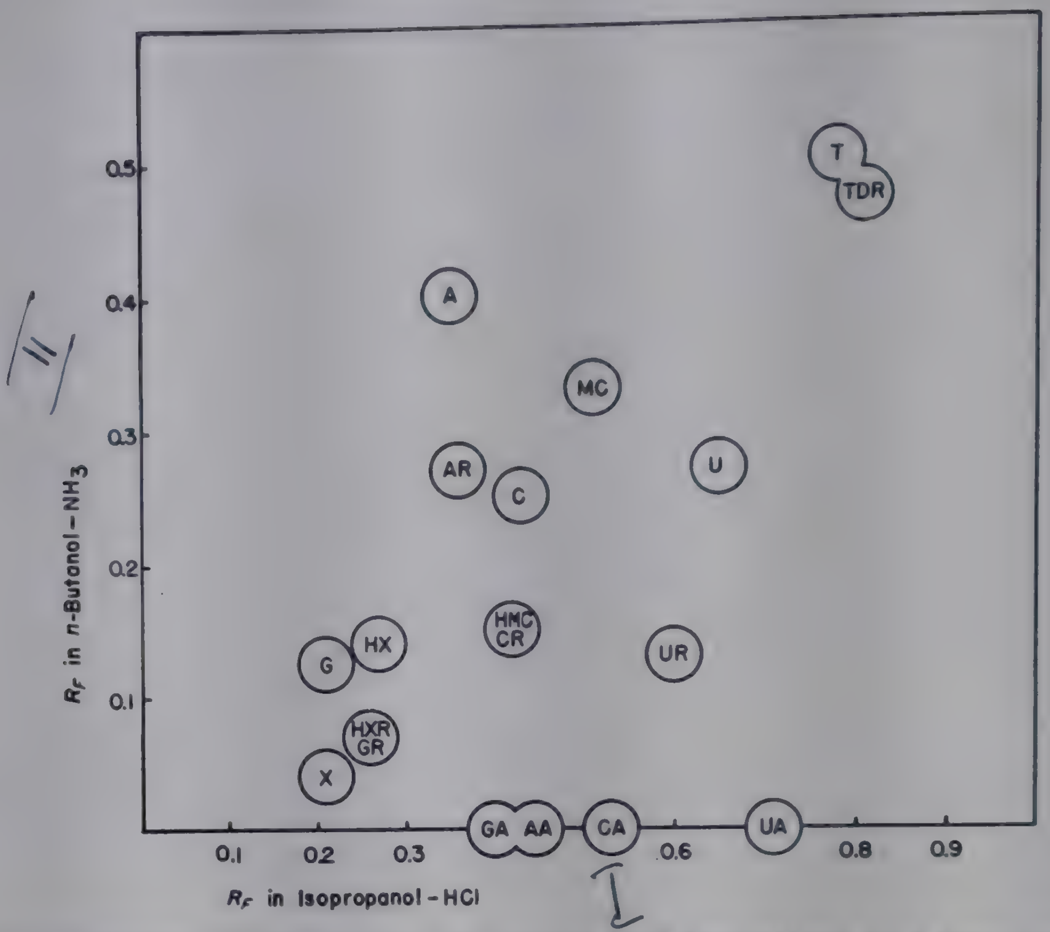

FIG. 1. Diagram of the positions of nucleic acid derivatives on a two-dimension chromatogram run on Whatman No. 1 paper by the descending technique first solvent *f* (Table I), then in solvent *c* (Table I; however, in the large chromatogra tank used here, the effective NH_3 concentration is reduced). A, adenine; AA, yea adenylic acid; AR, adenosine; C, cytosine; CA, cytidylic acid; CR, cytidine; guanine; GA, guanylic acid; GR, guanosine; HMC, 5-hydroxymethylcytosine; H hypoxanthine; HXR, inosine; MC, 5-methylcytosine; T, thymine; TDR, thymidin U, uracil; UA, uridylic acid; UR, uridine; X, xanthine.

This solvent resolves up to 75 μg. per spot of each of the DNA bases in 3 cm. movement of the front. Uracil is also resolved; 5-hydroxymethylcytosine, however, runs together with cytosine. Ribosides run at similar rate to their bases, and deoxyribosides rather faster; purine deoxyribosides ar decomposed by the acid.

When using solvents containing a high proportion of hydrochloric acid, the aci must be thoroughly removed from the paper at the conclusion of the run by evaporation at not too high a temperature (to avoid charring). Residual acid may damag photographic paper used for printing the chromatograms and, according to Schram and Kerekjarto,[63] on exposure to ultraviolet light may liberate chlorine which destroys cytosine by oxidation.

For mixtures too complex to be resolved in one dimension, a useful tw dimensional system is the isopropanol–HCl solvent in combination wit *n*-butanol–NH_3 or with isopropanol–NH_3. Better movement of guanine

[63] G. Schramm and B. von Kérekjártó, *Z. Naturforsch.* **7b**, 589 (1952).

obtained if the acid solvent is used first; the rate of flow of the second solvent s then more rapid than in untreated paper. The spacing of spots with such a system is shown in Figure 1.

Carter's dibasic sodium phosphate system is also included in Table I (solvent *i*). Pyrimidines are well separated from purines, but within each class there is little resolution. The use of water, or 0.01 *M* phosphate buffer, to bring about rapid separation of pyrimidines from purines was first described by Zamenhof *et al.*[64] Slightly greater dispersion of R_F values is given by water adjusted to pH 10, according to Levenbook,[65] whose data on this system are included in Table I (solvent *j*). The chief virtue of water as a solvent is its rapid flow, so that purine bases can be completely separated from pyrimidines in 3 hours.

3. Separation of Nucleotides

Because of the strong polarity of their phosphoryl groups, nucleotides do not move at appreciable rates in relatively nonpolar solvent systems uch as water-saturated *n*-butanol. Their movement may be accelerated by ncreasing the content of water or other polar components in the system, r by suppressing phosphoryl dissociation by addition of acid. These conditions prevent full advantage being taken of the ionic differences in the onstituent purine and pyrimidine bases, and separation of the nucleotides by paper chromatography has proven somewhat difficult. Effort has been xpended on the problem in a number of laboratories, and some of the more atisfactory solvents which have been developed are shown in Table II. None, however, is entirely satisfactory for separation of the nucleotides of he four bases from PNA in a single run, although these may be resolved by wo-dimensional chromatography or by electrophoresis on filter paper. Cf. *Smith*, Chapter 8.]

The two solvent systems containing hydrochloric acid (Table II, solvents *a* and both separate the pyrimidine ribonucleotides and the purine bases excellently from ne another. In solvent *a* cytosine and thymine deoxyriboside diphosphates are also resolved from the pyrimidine ribonucleotides.[66] The purine nucleotides are not satisfactorily resolved in either solvent. The isopropanol mixture is the faster running of the two. A mixture of *n*-butanol, ethanol, and 5 *N* HCl (3:2:2 by vol.) resolves purines and pyrimidine nucleosides and nucleotides, with the following R_F values;[67] guanine, 0.24; adenine, 0.35; cytidine, 0.43; cytidylic acid, 0.54; uridine, 0.63; uridylic acid, 0.78.

Solvent *c* is one of several mixtures of acetone with carboxylic acids tested by urrows *et al.*[39] It affords good separation of guanylic, cytidylic, and uridylic acids;

S. Zamenhof, G. Brawerman, and E. Chargaff, *Biochim. et Biophys. Acta* **9,** 402 (1952).

L. Levenbook, personal communication, 1953.

L. L. Weed and D. W. Wilson, *J. Biol. Chem.* **202,** 745 (1953).

J.-E. Edström, *Biochim. et Biophys. Acta* **9,** 528 (1952).

the R_F value of yeast adenylic acid is not recorded. Adenosine-5′-phosphate, aden sine diphosphate, and adenosine triphosphate are also separable from one another this system, or, with rather better spacing, in a mixture of 65 vol. of acetone with vol. of 15% trichloroacetic acid.

From the published R_F values, the buffered isoamyl alcohol–tetrahydrofurfur

TABLE II

R_F VALUES OF NUCLEOTIDES

	Solvent							
	tert-Butanol–HCl[a]	Isopropanol–HCl[b]	Acetone–trichloroacetic acid[c]	Isoamyl alc.–tetrahydrofurfuryl alc.–buffer[d]	Isobutyric acid–NH_3[e]	Phenol–*tert*-butanol–formic acid[f]	Na_2HPO_4–isoamyl alc.[g]	$(NH_4)_2SO_4$–buffer–isopropanol[h]
Adenosine-2′-phosphate[i]	0.50	0.48	—	0.35	0.49	0.70	0.74	0.26
Adenosine-3′-phosphate							0.67	0.16
Guanosine-2′-phosphate	0.46	0.43	0.20	0.67	0.24	0.46	0.79	0.50
Guanosine-3′-phosphate								0.40
Uridylic acid	0.80	0.77	0.51	0.43	0.24	0.35	0.85	0.73
Cytidylic acid	0.56	0.58	0.34	0.26	0.37	0.57	0.85	0.73
Adenosine-5′-phosphate	—	0.43	0.37	0.28	0.43	—	0.69	—
Adenosine diphosphate	—	—	0.10	0.07	—	—	0.77	—
Adenosine triphosphate	—	—	0.04	0.08	—	—	0.83	—
Deoxycytidylic acid	—	0.64	—	—	—	—	—	—
Thymidylic acid	—	0.81	—	—	—	—	—	—
Orthophosphate	0.90	0.84	0.61	—	—	0.22	—	—

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[a] *tert*-Butanol at 26° 700 ml., const.-boiling HCl 132 ml., water to make 1 liter; Whatman No. 1, descending; Smith and Markham, *Biochem. J.* **46**, 509 (1950). R_F values (20–22°) are from Boulanger and Montreuil, *Bull. soc. chim. biol.* **33**, 784, 791 (1951).

[b] See footnote *f*, Table I. The value for orthophosphate is from Markham and Smith, *Biochem. J.* **49**, 401 (1951).

[c] Acetone 75 vol., 25% (wt./vol.) trichloroacetic acid 25 vol.; Whatman No. 1 paper washed in 2 *N* HCl and water, ascending, at 4°; Burrows *et al.*, *Nature* **170**, 800 (1952).

[d] Isoamyl alc. 1 vol., tetrahydrofurfuryl alc. 1 vol., 0.08 *M* potassium citrate buffer (pH 3.02) 1 vol.; Whatman No. 1, descending, 20–25°; Carpenter, *Anal. Chem.* **24**, 1203 (1952).

[e] Isobutyric acid 10 vol., 0.5 *N* NH_4OH 6 vol., pH 3.6–3.7; Schleicher and Schüll No. 597, descending, 21–25°; Magasanik *et al.*, *J. Biol. Chem.* **186**, 37 (1950). R_F values calcd. from the published relative mobilities and figures.

[f] 90% aq. phenol 84 vol., *tert*-butanol 6 vol., formic acid 10 vol., water 100 vol.: after sepn. at a temp. 2–3° below that of the chromatography room, the nonaq. phase is used and the aq. phase is placed in the bottom of the tank; Whatman No. 1 paper, descending, 20–22°; Boulanger and Montreuil, *op. cit.*

[g] See footnote *i*, Table I. Values for adenosine-5′-phosphate, diphosphate, and triphosphate are from W. E. Cohn and C. E. Carter, *J. Am. Chem. Soc.* **72**, 4273 (1950).

[h] Satd. $(NH_4)_2SO_4$ in water 79 vol., 0.1 *M* buffer soln. (pH 6) 19 vol., isopropanol 2 vol.; Whatman No. 1 paper, descending; Markham and Smith, *op. cit.* R_F values are measured from the published figure.

[i] The "a" and "b" nucleotides of Carter, *J. Am. Chem. Soc.* **72**, 1466 (1950), and subsequent authors are now identified as the 2′-phosphates and 3′-phosphates, respectively, of the ribosides.

alcohol system of Carpenter[58] (solvent *d*) should give adequate separation of the nucleotides from PNA. Use of this mixture in analytical work has not yet been reported.

The isobutyric acid–ammonium isobutyrate solvent of Magasanik *et al.*[40] (solvent *e*) has been used for quantitative analysis of PNA's.[68] [Cf. *Magasanik*, Chapter 11.] Since guanylic and uridylic acids occupy the same position on the chromatogram, however, the amount of each must be calculated from extinction measurements taken at two wavelengths. It is reported[52a, 69] that the five deoxynucleotides from thymus DNA are completely resolved from one another in this solvent, the respective distances travelled (relative to deoxyadenylic acid taken as 100) being: deoxyguanylic acid, 53; thymidylic acid, 66; deoxycytidylic acid, 80; deoxyadenylic acid, 100; deoxy-5-methylcytidylic acid, 137.

The phenolic solvent system of Boulanger and Montreuil[70] (solvent *f*) is capable of separating the ribonucleotides with excellent spacing, and was used for quantitative analysis of PNA's. Because of the ultraviolet absorption of the phenol, however, it is necessary to detect and estimate the nucleotides by reactions of the phosphoryl group, and any other nucleic acid derivatives would of course be missed.

For fractionation of the products of digestion of PNA by ribonuclease, Markham and Smith[71, 72] have used (1) 70% (by vol.) aqueous isopropanol and (2) 70% aqueous isopropanol with concentrated ammonia added to the solvent in the bottom of the tank at the rate of 0.35 ml. for each liter of gas space. The latter system proved especially useful for initial fractionation (prior to electrophoresis or chromatography in other solvents) of the mixture of mono-, di-, and trinucleotides; in it the cyclic nucleotides (riboside-2′3′-monohydrogen phosphates) move more rapidly than the riboside-2′- and -3′-phosphates. It also resolves nucleotides from their benzyl esters.[73]

With systems based on salt solutions instead of organic solvents (Table II, solvents *g* and *h*), the purine ribonucleotides, but not those of the pyrimidines, have been separated. Carter's[22] sodium and potassium phosphate systems resolve adenosine-2′-phosphate and -3′-phosphate. The resolution of adenosine phosphates in dibasic sodium phosphate is said to be improved by substituting for isoamyl alcohol 0.5% lauryl amine in *n*-amyl alcohol.[74] The ammonium sulfate mixture of Markham and Smith[50] gives better spacing of the isomeric nucleotides and separates also those of guanylic acid and 8-azaguanylic acid;[30] in this solvent the cyclic nucleotides move more slowly than the corresponding nucleoside-2′- and -3′-phosphates.[71]

With water as the solvent, the R_F values of all the nucleotides are close to 0.9.[65]

V. Quantitative Estimation of the Nitrogenous Components of Nucleic Acids

1. Hydrolysis of Deoxypentose Nucleic Acids

The purine and pyrimidine components of deoxypentose nucleic acids are satisfactorily estimated as free bases, since these may be obtained in virtually quantitative yield by appropriate acid hydrolysis. Good yields o nucleosides or nucleotides have not been obtained from DNA by any mean of hydrolysis with acid or alkali. Nucleotides may be obtained enzymically,[7 but DNA containing 5-hydroxymethylcytosine resists this treatment[76] an chemical methods are preferable for routine work. [Cf. *Chargaff*, Chapter 10.

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[68] E. Chargaff, B. Magasanik, E. Vischer, C. Green, R. Doniger, and D. Elson, *J. Biol. Chem.* **186**, 51 (1950).

[69] E. Chargaff, *Federation Proc.* **10**, 654 (1951).

[70] P. Boulanger and J. Montreuil, *Bull. soc. chim. biol.* **33**, 784, 791 (1951).

[71] R. Markham and J. D. Smith, *Biochem. J.* **52**, 552 (1952).

[72] R. Markham and J. D. Smith, *Biochem. J.* **52**, 558, 565 (1952).

[73] D. M. Brown and A. R. Todd, *J. Chem. Soc.* **1953,** 2040.

[74] O. Snellmann and B. Gelotte, *Nature* **168,** 461 (1951).

a. Hydrolysis with hydrochloric acid

The purines are completely released from DNA by very mild acid treatment (pH 1.6 at 37° for 24 hr., or pH 2.8 at 100° for 1 hour[77]). More drastic hydrolysis with hydrochloric acid (10 mg. nucleic acid in 2 ml. 6 *N* HCl at 120° for 2 hours[78, 79]) gives a good yield of pyrimidines but causes some destruction of purines. Nearly quantitative yields of all the bases (including 5-hydroxymethylcytosine but with evidence for slight destruction of adenine) have been obtained by Hershey *et al.*[57] by subjecting the partially hydrolyzed nucleic acid extracted from bacteriophage T2 with hot trichloroacetic acid to further hydrolysis with 3 ml. redistilled 6 *N* HCl under CO_2 in sealed tubes at 100° for 3 hours.

b. Hydrolysis with perchloric acid

The use of concentrated perchloric acid for liberation of bases from nucleic acids was introduced by Marshak and Vogel.[53, 80] With DNA, either 7.5 *N* or 12 *N* (70%) $HClO_4$ may be used, and maximal yields are obtained in 1 hour at 100°. Loss of a small percentage of the thymine has been noted during hydrolysis with 70% $HClO_4$, which may be minimized by using not more than 15 μl. $HClO_4$ per mg. DNA, although if much protein is present the amount may be increased.[81] 5-Hydroxymethylcytosine is extensively destroyed during hydrolysis of bacteriophage DNA with perchloric acid, although the isolated pyrimidine is apparently not attacked by this acid.[11, 57] By hydrolyzing with $HClO_4$ it is possible to estimate the total purine and pyrimidine bases from a mixture containing both DNA and PNA, and to estimate the bases without isolation of nucleic acid from some biological materials. Non-nucleic acid components are degraded to products which interfere very little on the chromatograms. Perchloric acid hydrolysates may be diluted with water and applied directly to paper for chromatography. If phosphorus is to be estimated in samples of the hydrolysate, the insoluble residue from the nucleic acid carbohydrate, which tends to adsorb phosphate, should first be brought into suspension.[82]

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[75] R. O. Hurst, J. A. Little, and G. C. Butler, *J. Biol. Chem.* **188,** 705 (1951).

[76] S. S. Cohen and G. R. Wyatt, unpublished results, 1953

[77] C. Tamm, M. E. Hodes, and E. Chargaff, *J. Biol. Chem.* **195,** 49 (1952).

[78] M. M. Daly, V. G. Allfrey, and A. E. Mirsky, *J. Gen. Physiol.* **33,** 497 (1950).

[79] N. I. Gold and S. H. Sturgis, *J. Biol. Chem.* **196,** 143 (1952).

[80] A. Marshak and H. J. Vogel, *Federation Proc.* **9,** 85 (1950).

[81] G. R. Wyatt, *J. Gen. Physiol.* **36,** 201 (1952).

[82] Levenbook[65] has obtained evidence for differential adsorption of purine and pyrimidine bases also to the charcoal from nucleic acid samples contaminated with carbohydrates.

c. Hydrolysis with formic acid

The value of formic acid for liberating purine and pyrimidine bases from nucleic acids without destruction was first shown by Vischer and Chargaff,[83] and this acid has proved very satisfactory for the quantitative hydrolysis of DNA. The conditions used in Chargaff's laboratory have been 98–100 % formic acid at 175° for 2 hours. Shorter periods of heating have also been employed.[47, 55] Recently,[11] good results were obtained with DNA from bacteriophages and other sources using 88 % formic acid at 175° for 30 minutes, and it was found that whether 88 % or 98 % formic acid is used, recoveries of guanine and hydroxymethylcytosine are substantially increased by using a relatively large volume of it (500 μl. per mg. DNA) and by avoiding excessive oxidizing atmosphere while heating. The hydrolysis is carried out in sealed Pyrex glass bomb tubes, and decomposition of the formic acid produces considerable pressure, which it is advisable to release by melting the tips of the tubes in a flame before opening them. The hydrolysate is evaporated under reduced pressure to dryness, then redissolved in a small volume of *N* HCl, and samples are taken for chromatography and for estimation of phosphorus. Because of the high recoveries and the absence of insoluble residue in the hydrolysate, this is probably the method of choice for accurate microanalysis of purified preparations of DNA.

2. Hydrolysis of Pentose Nucleic Acids

The ribosides of both purines and pyrimidines are split with more difficulty than their deoxyribosides, and the resistance of the pyrimidine ribosides is such that it is very difficult to get quantitative yields of the free bases from PNA. However, the ease with which mononucleotides are obtained from PNA makes it possible to estimate the pyrimidines, or all of the bases, as nucleotides. [Cf. *Loring*, Chapter 5, and *Magasanik*, Chapter 11.]

a. Hydrolysis to purine and pyrimidine bases

Concentrated formic acid at 175° for 2 hours has been used for liberation of pyrimidines from PNA;[83] however, the yield of uracil is very low and the method has been found unsatisfactory for quantitative hydrolysis of uridylic acid.[68] Nearly quantitative yields of all the bases can be obtained with 70–72% perchloric acid at 100° for 1 hour,[47, 53] and this method has been used for analysis of PNA's.[84]

b. Hydrolysis to purine bases and pyrimidine nucleotides

The purine bases are liberated from PNA by relatively mild acid hydrolysis, e.g., *N* H_2SO_4[83] or *N* HCl[85] at 100° for 1 hour. The latter treatment was selected by Smith and Markham for quantitative analysis of PNA's: pyrimidines are estimated as nucleotides, a correction of 5% being applied to allow for degradation to nucleosides.[5] Abrams,[86] using isotope dilution to test comparable hydrolytic conditions, has demonstrated about 7% destruction, probably deamination, of adenine and guanine.

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[83] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176,** 715 (1948).

[84] A. Marshak, *J. Biol. Chem.* **189,** 597 (1951).

[85] Smith and Markham[29] include a critical discussion of previously used methods of hydrolysis.

c. *Hydrolysis to nucleotides*

As has long been known, PNA, unlike DNA, is readily hydrolyzed to mononucleotides. [Cf. Chapter 11, and also *Brown and Todd*, Chapter 12.] This may be achieved with N NaOH or N HCl at room temperature; the latter is less convenient because the nucleic acid dissolves only slowly and there is danger of splitting purine glycosidic linkages.[50] Chargaff *et al.*[68] used pH 13 to 14 at 30° overnight to degrade PNA to nucleotides prior to chromatographic separation. Boulanger and Montreuil[70] used 0.5 N NaOH at 20–22° for 18 hours, or concentrated ammonia solution ($D^\circ = 0.925$) at 45° for 8 days, with good results, although ammonia under other conditions is liable to produce nucleosides. At 37°, N alkali causes partial deamination of cytidylic acid, but 0.3 N alkali does not.[87] This was confirmed by Davidson and Smellie,[88] who used 0.3 N KOH at 37° for 18 hours to convert PNA to nucleotides, the potassium being removed as the perchlorate prior to separation of nucleotides by electrophoresis on filter paper. Crosbie *et al.*[89] analyzed a number of samples of PNA by this method as well as by hydrolysis in N HCl and in concentrated $HClO_4$ in conjunction with paper chromatography: alkaline hydrolysis gave consistently higher results for uracil, for cytosine, and for total recovery in terms of phosphorus than either of the other procedures. Alkaline hydrolysis followed by electrophoretic separation of nucleotides appears to be the most reliable method yet devised for microanalysis of PNA. [Cf. *Smith*, Chapter 8.]

3. QUANTITATIVE TECHNIQUE

Small volumes of hydrolysates or other solutions can be measured on to filter paper for quantitative chromatography with a variety of micropipets and burets. Especially convenient are self-filling capillary pipets,[90] which are not difficult to make; those available commercially often have excessively thick tips and a bore so fine that they are liable to clogging and drainage error. The volume which may be placed on the paper in one application depends on the scale of working: in the range of 10 to 50 μg. of each purine or pyrimidine base, to be run in one dimension for about 18 hours, a suitable volume is about 10 μl., and using less does not result in significantly smaller final spots.

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[86] R. Abrams, *Arch. Biochem.* **30,** 44 (1951).

[87] D. H. Marrian, V. L. Spicer, M. E. Balis, and G. B. Brown, *J. Biol. Chem.* **189,** 533 (1951).

[88] J. N. Davidson and R. M. S. Smellie, *Biochem. J.* **52,** 594 (1952).

[89] G. W. Crosbie, R. M. S. Smellie, and J. N. Davidson, *Biochem. J.* **54,** 287 (1953).

[90] P. L. Kirk, "Quantitative Ultramicroanalysis," p. 22. John Wiley and Sons, New York, 1950.

A substance may be efficiently eluted in a small volume by cutting out a pointed band of paper including the spot, and allowing water to diffuse through it toward the point. The solute is carried with the water, which may be collected from the point of the paper in a small vessel,[91] in a pipet[92] or directly on another sheet of filter paper for re-chromatography.[93] This technique has been used to concentrate minor components such as 5-methylcytosine from nucleic acids for estimation.[48]

For quantitative estimation, spots are cut out and eluted by soaking in a volume of liquid appropriate for the cells in which the extinctions are to be read (4–5 ml. for the 1-cm. cells of the Beckman or similar spectrophotometer). 0.1 *N* HCl is an eluent[17] in which the nucleic acid bases are sufficiently soluble, and in which some of them have higher extinction coefficients than in neutral or alkaline solutions. Standing overnight at room temperature, with shaking before and after, effects quantitative elution. To allow for ultraviolet-absorbing substances in the paper, blanks are cut equal in area to the spots and at equal distances from the starting line, and are eluted and read at the same wavelengths as the corresponding spots. As a further precaution against error due to ultraviolet-absorbing contaminants, Vischer and Chargaff[17] read the extinctions of all eluates both at the absorption peak of the substance being estimated and at another reference wavelength where its absorption is low, basing their calculations on the difference. The same procedure was adopted by Crosbie *et al.*[89] in analyzing HCl and $HClO_4$ hydrolysates, and it is evident that it will generally reduce the error from contaminants, although their absorption at the two wavelengths will rarely be precisely equal. Spectral data for use in estimating nucleic acid derivatives by the absorption at their maxima and by the difference method are given in Tables III and IV, respectively. [Cf. *Beaven, Holiday and Johnson*, Chapter 14.] Hotchkiss[2] gives tables of the absorption of nucleic acid derivatives at 5-mμ wavelength intervals, which may be used to compute the composition of binary mixtures from density readings at two or more wavelengths.

The methods which have been described are suitable for estimation of purine and pyrimidine derivatives in the range of 5 to 100 μg. per spot. Quantitative analyses in duplicate have been conveniently carried out on samples of 0.3 to 1.0 mg. of nucleic acid by making up hydrolysates to a volume of 25 μl., from which two 8-μl. portions are taken for chromatography and two 2-μl. portions for phosphorus estimation.[11, 81] In an ultra-micro adaptation of paper chromatography recently described by Edström,[67] only one hundredth of this amount of material is required. The hydrolysate

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[91] C. E. Dent, *Biochem. J.* **41,** 240 (1947).

[92] R. Consden, A. H. Gordon, and A. J. P. Martin, *Biochem. J.* **41,** 590 (1947).

[93] A. M. Moore and J. B. Boylen, *Science* **118,** 19 (1953).

TABLE III

ULTRAVIOLET ABSORPTION DATA ON PURINE AND PYRIMIDINE DERIVATIVES

Substance	Normality of HCl	Wavelength, mμ[a]	Millimolar extinction coefficient	$E_{1\,\mathrm{cm.}}^{0.001\%}$
Adenine[47]	0.1	260	13.0	0.96
Guanine[47]	0.1	250	11.0	0.73
Uracil[47]	0.1	260	7.9	0.705
Thymine[47]	0.1	265	7.95	0.63
Cytosine[47]	0.1	275	10.5	0.95
5-Methylcytosine[47]	0.1	283	9.8	0.785
5-Hydroxymethylcytosine[11]	0.1	279	9.7	0.685
Adenylic acid[94]	0.01	260	13.9	0.401
Guanylic acid [94]	0.01	260	11.8	0.325
Uridylic acid[95]	0.01	262	9.89	0.305
Cytidylic acid[95]	0.01	278	12.72	0.393

[a] Not, in all cases, the precise absorption maximum.

TABLE IV

ULTRAVIOLET ABSORPTION DATA ON PURINE AND PYRIMIDINE DERIVATIVES

Substance	Solvent	Absorption Maximum, mμ	Reference wavelength, mμ	Difference in $E_{1\,\mathrm{cm.}}^{0.001\%}$ at the two wavelengths
Adenine[89]	0.1 *N* HCl	262.5	290	0.935
Guanine[89]	1.6 *N* HCl	249	290	0.539
Cytosine[89]	0.1 *N* HCl	275	290	0.546
Uracil[89]	0.1 *N* HCl	259	280	0.609
Thymine[17]	Water	264.5	290	0.545
Cytidylic acid[89]	0.01 *N* HCl	278	300	0.331
Uridylic acid[89]	0.1 *N* HCl	260.5	280	0.814

of 1.5 to 5 μg. of nucleic acid is applied in a volume of about 0.5 μl. to a strip of filter paper 0.5 to 1 mm. broad, which is subjected to chromatography in the ordinary way. A contact print of the dried strip chromatogram is made on a process plate with light of wavelengths 257 and 275 mμ from rotating cadmium electrodes, then the darkening of the plate is measured with a recording microphotometer, the image of a rotating sector serving for calibration. This refined technique will undoubtedly be of value in certain biological problems; however, the equipment is not available in the average laboratory, and one may note that by using narrow strips of paper as described by Edström to prevent spreading of the spots, and eluting the bands for extinction measurement in microcells[96] on the spectrophotometer, comparable sensitivity could

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[94] E. Volkin and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1516 (1951).

[95] J. McT. Ploeser and H. S. Loring, *J. Biol. Chem.* **178,** 431 (1949).

[96] O. H. Lowry and O. A. Bessey, *J. Biol. Chem.* **163,** 633 (1946).

TABLE V

R_F VALUES OF PENTOSES

	Solvent			
	Phenol[a]	Methyl ethyl ketone[b]	Butanol–ethanol[c]	Butanol–pyridine[d]
Arabinose	0.54	0.075	0.12	—
Xylose	0.44	0.090	0.15	—
Lyxose	—	0.125	0.19	—
Ribose	0.59	0.165	0.21	0.49
Rhamnose	0.59	0.180	0.30	0.56
2-Deoxyribose	0.73	—	0.44	0.60

[a] Phenol satd. with water at 20°, with 1% NH_3 and a few crystals KCN in soln. in bottom of tank; Whatman No. 1 paper; Partridge, *Biochem. J.* **42,** 238 (1948).

[b] Methyl ethyl ketone satd. with water at 20°, with 1% NH_3 in soln. in bottom of tank; Whatman No. 1; Partridge, *op. cit.* The value for lyxose is calcd. from the data of Crosbie *et al.*, *Biochem. J.* **54,** 287 (1953).

[c] *n*-Butanol 50 vol., ethanol 10 vol., water 40 vol., the upper layer being used; Whatman No. 1. R_{TG} values (movement relative to that of 2,3,4,6-tetramethylglucose taken as 1.00) are shown. E. L. Hirst and J. K. N Jones, *Discussions Faraday Soc.* **7,** 271 (1949).

[d] To the upper layer resulting from the mixture of 1 vol. pyridine, 1.5 vol. water, and 3 vol. *n*-butanol, at about 28°, is added 1 vol. pyridine; Schleicher and Schüll No. 597 paper. Chargaff *et al.*, *J. Biol. Chem.* **177,** 405 (1949).

be achieved. The bases from 5 μg. of yeast PNA, each eluted in 0.1 ml. and examined in a cell of 1 cm. path length, would give optical densities in the range 0.25 to 0.5, so that it should be entirely practical to work on this scale without special equipment.

VI. Chromatography of Nucleic Acid Sugars

This account would be incomplete without reference to the identification of nucleic acid carbohydrates by paper chromatography. However, since the methods do not differ from those used for other carbohydrates, which have been adequately reviewed,[4-6, 97] and since the results with nucleic acid components have been limited in scope, this description will be kept very brief.

Numerous spray reagents have been described for detection of sugars on paper chromatograms. Ammoniacal silver nitrate, used in the original investigations in this field,[98] is sensitive but relatively nonspecific and subject to interference by impurities. *m*-Phenylenediamine[99] forms with a wide range of aldoses and ketoses derivatives which fluoresce in ultraviolet light, and has been used in Chargaff's laboratory for recognition of nucleic acid sugars. Aniline hydrogen phthalate[100] is a sensitive and convenient reagent

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[97] S. M. Partridge, *Biochem. Soc. Symposia* (*Cambridge, Engl.*) **No. 3,** 52 (1949).

[98] S. M. Partridge, *Biochem. J.* **42,** 238 (1948).

[99] E. Chargaff, C. Levine, and C. Green, *J. Biol. Chem.* **175,** 67 (1948).

[100] Aniline, 0.93 g., and phthalic acid, 1.66 g., are dissolved in 100 ml. water-saturated butanol. The papers are sprayed and then heated to 105° for 5 min. S. M. Partridge, *Nature* **164,** 443 (1949).

for aldoses, including ribose and deoxyribose, but not for ketoses; increase range of sensitivity is reported for aniline hydrogen phosphate.[101] The reagents mentioned earlier (Section III.2) for ribosides and deoxyriboside react also with the free sugars.

The R_F values of the pentoses, along with rhamnose and 2-deoxyribose in several chromatographic solvents are shown in Table V. Improved separations of a number of sugars are reported by Jermyn and Isherwood[10 using ethyl acetate–pyridine–water (2:1:2) and ethyl acetate–acetic acid–water (3:1:3) as solvents; however, R_F values of ribose and deoxyribose were not determined. 2-Deoxyribose, 3-deoxyribose, and 4-deoxyribose are separable with a butanol–ethanol mixture.[103] The D- and L-isomers of sugars have not been separated on paper chromatograms.

The purine-bound sugar from pentose nucleic acids may be liberated by hydrolysis in *N* H_2SO_4 at 100° for 1 hour,[83] and the hydrolysate can be applied to paper for chromatography without removal of the acid.[104] From DNA, because of the lability of the sugar and the stability of the internucleotide linkages, enzymic hydrolysis is required: Chargaff *et al.*[105] used deoxyribonuclease together with a phosphatase derived from *Aspergillus* to give nucleosides, after which the purine nucleosides were split by heating at pH 1.5 to 100° for 12 minutes.

The sugar components of all pentose nucleic acids yet examined by these methods have proved to be chromatographically identical with ribose, and those of deoxypentose nucleic acids, with 2-deoxyribose. [Cf. Chapters 2, 10, and 11.]

VII. Addendum

The following reports have appeared, or have come to the writer's attention, since preparation of this chapter.

A solvent composed of 7 vol. 95% ethanol plus 3 vol. *M* ammonium acetate buffer of pH 3.8, used in the study of uridine diphosphate glucose, gives fair resolution of the nucleotides of PNA.[106] Several systems suitable for resolution of deoxyribosides have been described by Tamm *et al.*[52a] Caldwell[107] has demonstrated resolution of adenosine-5′-phosphate, diphosphate, and triphosphate with Hanes and Isherwood's solvent containing 60 cc.

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[101] *N* Aniline in butanol, 1 vol., mixed with 2 *N* H_3PO_4 in butanol, 2 vol.; J. L. Bryson and T. J. Mitchell, *Nature* **167**, 864 (1951).

[102] M. A. Jermyn and F. A. Isherwood, *Biochem. J.* **44**, 402 (1949).

[103] P. F. V. Ward and P. W. Kent, *Nature* **170**, 936 (1952).

[104] B. D. E. Gaillard, *Nature* **171**, 1160 (1953).

[105] E. Chargaff, E. Vischer, R. Doniger, C. Green, and F. Misani, *J. Biol. Chem.* **177**, 405 (1949).

[106] A. C. Paladini and L. F. Leloir, *Biochem. J.* **51**, 426 (1952).

[107] P. C. Caldwell, *Biochem. J.* **55**, 458 (1953).

-propanol, 30 cc. concentrated ammonia solution, and 10 cc. water. He has lso found useful for detection of ultraviolet absorbing spots a sheet of paper mpregnated with proflavine as a fluorescent screen.

The circular method of paper chromatography of Rutter[108] and others, or which improved resolution is claimed, has been applied to nucleic acid erivatives.[109]

A comprehensive tabulation of ultraviolet absorption data, including olar extinction coefficients and the ratios of extinctions at different wave-engths, measured at three pH values, has been prepared by Volkin and ohn.[110]

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[108] L. Rutter, *Nature* **161,** 435 (1948).

[109] K. V. Giri, P. R. Krishnaswamy, G. D. Kalyankar, and P. L. N. Rao, *Experientia* **9,** 296 (1953).

[110] E. Volkin and W. E. Cohn, *in* "Methods of Biochemical Analysis," (D. Glick, ed.) Vol. 1, Interscience Publishers, New York (1954), and *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 2, Academic Press, New York (in press).

CHAPTER 8

The Electrophoretic Separation of Nucleic Acid Components

J. D. SMITH

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Nucleic acids and their components bear a number and variety of ionizable groups; and it is natural that electrophoretic techniques should play an important part in their separation. The development of electrophoresis on paper has greatly facilitated separations of nucleic acid derivatives which would be impossible by paper chromatography and time-consuming by ion-exchange methods. All the manipulations can be carried out on very small amounts of material. An important feature of the method is that the relative mobilities of nucleic acid components at any given pH may be predicted quite accurately, thus aiding in the identification of unknown components.

I. Theory of the Electrophoretic Separation of Nucleic Acid Components

1. General Considerations

A molecule in a fluid subjected to a voltage gradient (E) is acted upon by a force equal to (EQ), where Q is the net charge on the molecule and is given by the algebraic sum of the products of the number of ionizing groups and their percentage dissociation. This force is opposed by that due to th resistance of flow of the molecule through the liquid, which is proportiona to the velocity of motion of the molecule (v) and equal to (Kv), where K is function of the size and shape of the molecule and the retarding effects o other ions in the solution. The molecules thus migrate with a constan velocity equal to (EQ/K).

Using the dissociation constants of the ionizable groups in nucleic aci components and making certain approximations as to the nature of th resistance to flow of the charged molecules through the fluid, the relativ mobilities of these substances may be calculated. This is best illustrated i the case of nucleotides and polynucleotides.

2. Separation of Nucleotides and Polynucleotides

The ionizing groups in nucleotides and polynucleotides are the primary and secondary phosphate groups, the amino groups of adenine, guanine and cytosine and its derivatives, and the enol groups of guanine, cytosine uracil, and thymine. Table I gives the pK values of these groups for a number of nucleotides. [For a more complete discussion, compare *Jordan*, Chapter 13.] The dissociation constants of the pairs of isomeric ribonucleoside-2′- and -3′-phosphates are very close to each other, so that all calculations have been made with the values of Levene and Simms[1-4] which are probably based on mixtures of the two isomers. The dissociation constants of the deoxyribonucleotides have not been determined, but to a close approximation they may be assumed to be identical with those of the corresponding ribonucleotides. In Figure 1 are plotted the dissociation curves for the ionizing groups of the four nucleotides, adenylic, guanylic, cytidylic, and uridylic acids.

The enol groups of guanylic, cytidylic, and uridylic acids having pK values of 9.5 or above are not dissociated at the pH values useful for most separations, and so for the moment may be ignored, but the primary and secondary phosphate groups and the amino groups are most important. The pK values of the primary phosphate dissociations which lie between 0.7 and 1.0 are too close to each other to be used for the separation of the nucleotides; and also some nucleotides and most polynucleotides are unstable in this pH range. The separation of adenylic, guanylic, cytidylic, and uridylic acids is possible on the basis of the differences in the pK values of the amino groups, which lie between 2 and 5. In this pH range the primary phosphate groups are completely dissociated and the secondary phosphate groups uncharged. The most suitable pH for the separation is

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[1] P. A. Levene and H. S. Simms, *J. Biol. Chem.* **65**, 519 (1925).

[2] P. A. Levene, L. W. Bass, and H. S. Simms, *J. Biol. Chem.* **70**, 229 (1926).

[3] P. A. Levene, H. S. Simms, and L. W. Bass, *J. Biol. Chem.* **70**, 243 (1926).

[4] P. A. Levene and H. S. Simms, *J. Biol. Chem.* **70**, 327 (1926).

TABLE I

THE DISSOCIATION CONSTANTS OF SOME PURINES, PYRIMIDINES, NUCLEOSIDES, AND NUCLEOTIDES[a]

Substance	(NH_2)	First (OH)	Primary phosphate	Secondary phosphate
Cytosine[2]	4.60	12.16	—	—
Uracil[2]	—	9.45	—	—
Thymine[2]	—	9.94	—	—
Adenosine[3]	3.3	—	—	—
Inosine[3]	—	8.75	—	—
Guanosine[1]	1.6	9.16	—	—
Cytidine[1]	4.22	12.3	—	—
Uridine[1]	—	9.17	—	—
Adenylic acid[1]	3.70	—	0.89	6.01
Adenosine-5′-phosphate[5]	3.80	—		6.2
Inosine-5′-phosphate[3]	—	8.88	1.54	6.04
Guanylic acid[1]	2.3	9.7	0.70	5.92
Cytidylic acid[1]	4.24	13.2	0.80	5.97
Cytidylic acid[6] (2′-phosphate)	4.36			
Cytidylic acid[6] (3′-phosphate)	4.28			
Uridylic acid[1]	—	9.43	1.02	5.88

[a] The values given under the headings adenylic, guanylic, cytidylic, and uridylic acids[1] were probably determined on mixtures of the 2′ and 3′-phosphates. The amino group p*K* given for adenosine-5′-phosphate[5] does not bear the expected relation to that of the mixture of adenosine-2′- and -3′-phosphates and may be ncorrect.

3.5 where the degree of dissociation of the NH_2 group of adenylic acid is 0.54, guanylic acid 0.05, cytidylic acid 0.84, while uridylic acid of course has none. As each nucleotide carries a negative charge of 1 due to the primary phosphate group, the net negative charges are: adenylic acid 0.46, guanylic acid 0.95, cytidylic acid 0.16, and uridylic acid 1.00. The four mononucleotides have very nearly the same size and so approximately the same resistance to motion; consequently these figures give their relative mobilities at this pH. With the use of the dissociation curves the relative mobilities of the nucleotides may be calculated for any other pH.

The p*K* values of the amino groups of the isomeric nucleoside-2′-, -3′-, and -5′-phosphates differ so little that they would only be expected to show separation in a long electrophoretic run and in practice generally run together, although Davidson *et al.*[7, 8] have observed incomplete separation of guanosine-2′- and -3′-phosphates at pH 3.5.

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[5] H. Wassermeyer, *Z. physiol. Chem.* **179,** 238 (1928).

[6] L. F. Cavalieri, *J. Am. Chem. Soc.* **74,** 5804 (1952).

[7] J. N. Davidson and R. M. S. Smellie, *Biochem. J.* **52,** 599 (1952).

[8] G. W. Crosbie, R. M. S. Smellie, and J. N. Davidson, *Biochem. J.* **54,** 287 (1953).

It might be thought that a mononucleotide and the corresponding dinucleotide, which below pH 5 has twice the charge and approximatel twice the size, would not differ in electrophoretic mobility. This is not th

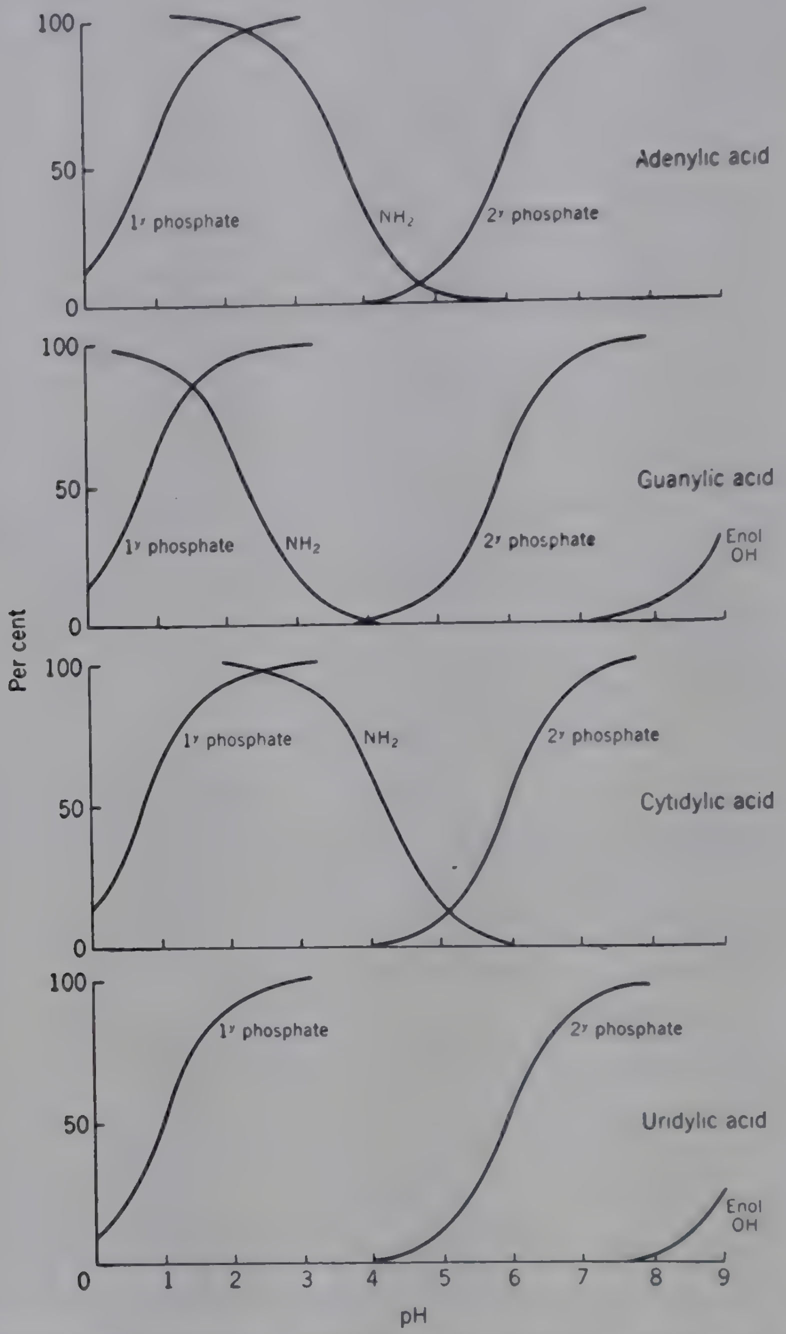

FIG. 1. The dissociation curves of the ionizing groups of adenylic, guanylic, cytidylic, and uridylic acids plotted from the data of Levene and Simms.[1]

case, for the separation of polynucleotides depends on the relative resistances to motion of a mononucleotide, its dimer, trimer, etc. Before attempting the electrophoretic separation of polynucleotides, Markham and Smith[9, 10] calculated the probable limits that could be set to the relative resistance to motion of a mononucleotide and its dimer. From its diffusion

[9] R. Markham and J. D. Smith, *Nature* **168,** 406 (1951).

[10] R. Markham and J. D. Smith, *Biochem. J.* **52,** 558 (1952).

constant[11, 12] adenylic acid would appear to have an effective axial ratio of about 5:1. The axial ratio of the dinucleotide must thus lie between 2.5:1 and 10:1. Taking the shape to be an oblate ellipsoid, and assuming the resistance to motion to be the same as in translational diffusion, the ratio of the frictional resistance of the dimer to that of the monomer lies between the limits 1.32 and 1.85. The observed relative mobilities on paper of the mono-, di-, and trinucleotides of uridylic acid (after correction for movement due to flow of the buffer solution through the paper) are 1:1.4:1.5 at pH 3.5.[10] At this pH the net charges on the mono-, di-, and triuridylic acids are 1, 2, and 3 so that if the frictional resistance to motion of a mononucleotide is taken as 1, that of a dinucleotide is 1.43 and of a trinucleotide 2.0.

Using these figures and the dissociation curves, the mobilities of any mono-, di-, or trinucleotide relative to uridylic acid may be calculated for any pH. For example at pH 3.5 the relative mobilities of

$$\begin{aligned} \text{AG} &= (0.46 + 0.95)/1.43 &&= 0.99 \\ \text{of AC} &= (0.46 + 0.16)/1.43 &&= 0.43 \\ \text{and of AAU} &= (0.46 + 0.46 + 1.0)/2 &&= 0.96 \end{aligned}$$

times that of uridylic acid. (Here A, G, C, and U represent adenylic, guanylic, cytidylic, and uridylic acid residues, respectively.) It is again assumed that the mononucleotides have approximately the same size and shape, and that the dissociation constants of individual groups are the same, whether in nucleotides or in polynucleotides.

Evidently the relative mobilities of the mono-, di-, and trimers of a single nucleotide form a converging series so that separation of the tetra- and higher polynucleotides by this method is unlikely.

Remarkably good agreement is found between the mobilities relative to uridylic acid calculated on this basis and those observed in practice with the technique of electrophoresis on paper immersed in carbon tetrachloride (Section II). In Figure 2 are plotted the observed movements of 19 mono- and polynucleotides in 0.05 M ammonium formate buffer pH 3.5 against their calculated mobilities relative to that of uridylic acid. These data were taken from preparative runs in which no particular care was taken to control the voltage gradient to better than ±10%. Under these conditions about 25% of the movement of uridylic acid is accounted for by flow of the buffer solution due to endosmosis and incomplete saturation of the paper. Table II gives the calculated relative mobilities and observed movement of a number of nucleotides and polynucleotides at pH 3.5.

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[11] A. H. Gordon and P. Reichard, *Biochem. J.* **48,** 569 (1951).

[12] G. Schramm, W. Albrecht, and K. Munk, *Z. Naturforsch.* **7b,** 10 (1952).

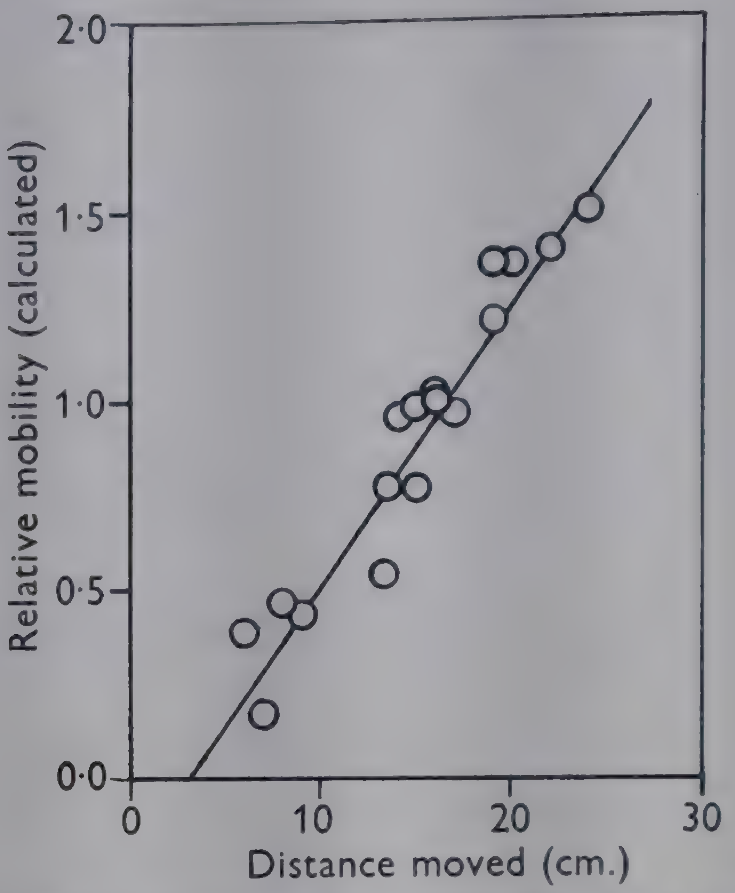

FIG. 2. Graph of mobility observed (in cm./2 hr./20 v./cm.) against the mobili (relative to uridylic acid) calculated from the charge and frictional resistance f 19 mono- and polynucleotides[10] (two of the points are on top of others).

While 3.5 is one of the most useful pH values, the four nucleotides havir the greatest differences in net charge at pH 3.5, it is not suitable for a separations. Another useful pH is 5 where all mono-, di-, and trinucleotid have net negative charges of approximately 1, 2, and 3, respectively, an so separate with relative mobilities of 1:1.4:1.5. It is important to no that at pH 7–8, where the secondary phosphate is fully dissociated, th mono-, di-, and trinucleotides have relative charges of 2, 3, and 4 and the relative mobilities are 1, 1.05, and 1 so that in this pH range their separatio is not possible.

3. Purines, Pyrimidines, and Nucleosides

The ionizing groups on the bases and nucleosides are the amino and en groups (the basic dissociations of the purine rings have pK values betwee 0 and 1[13] and may be neglected). The dissociation constants of these ar close to, although not identical with, those of the corresponding nucleotide (Table I), so their electrophoretic separation is similar to that of the cor responding nucleotides. At pH 3.5, for example, uridine is uncharge while cytidine, adenosine, and guanosine migrate towards the cathode i

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[13] J. K. Wood, J. Chem. Soc. **89**, 1839 (1906).

TABLE II

HE CALCULATED MOBILITIES RELATIVE TO URIDYLIC ACID OF SOME NUCLEOTIDES ND POLYNUCLEOTIDES AT pH 3.5 AND THEIR OBSERVED MOVEMENT IN 0.05 *M* AMMONIUM FORMATE BUFFER pH 3.5 AT 20 v./cm.[10] THE RELATIVE POSITIONS OF THESE SUBSTANCES ON PAPER CHROMATOGRAMS IN SOLVENT 1 ARE ALSO GIVEN (THE BANDS ARE NUMBERED 1–6 IN ORDER OF INCREASING R_F VALUE).

Substance	Calculated mobility relative to that of uridylic acid	Observed movement in 0.05 *M* ammonium formate buffer pH 3.5 (cm./2 hr. at 20 v./cm.)	Position on the chromatogram in solvent 1 (bands numbered in order of increasing R_F value)
A	0.46	8	band 4
G	0.95	14	2
C	0.16	6.5	4
U	1.00	16	4
A!	0.46	8	6
G!	0.95	14	5a (between 5 and 6)
C!	0.16	7	6
U!	1.00	16	6
AC!	0.43	9	5
AU!	1.02	16	5
UU!	1.4	22	5
AC	0.43	8	2
AU	1.02	16	2
AG	0.99	15	1
GC!	0.78	15	3
GU!	1.36	20	3
GC	0.78	13.5	1
GU	1.36	19.5	1
UUU!	1.50	24	5
ACC!	0.39	6	2
AAC	0.54	13.5	1
AAU	0.96	17	1
AGU	1.21	19	1

[a] A, G, C, and U denote adenylic, guanylic, cytidylic, and uridylic acids, respectively. The symbol ! is used to indicate a 2′,3′-monohydrogen phosphate group.

the reverse order to that in which the mononucleotides move towards the anode. At this pH the purines and pyrimidines move in the same order as the nucleosides. These separations have been described by Dimroth *et al.*,[14] but the order they report for the migration of the bases at pH 3.2 is not that expected theoretically, nor is it found in practice.

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[14] K. Dimroth, L. Jaenicke, and I. Vollbrechtshausen, *Z. physiol. Chem.* **289,** 71 (1952).

II. Apparatus and Techniques

The first attempt at the separation of nucleic acid components by electr phoresis was made by Gordon and Reichard,[11] who ran deoxyribonucleas digests of deoxyribonucleic acid on agar gel. The use of agar as a supportin medium has obvious disadvantages. It is difficult to avoid overheating an consequent "sweating" of the gel, and the recovery of the separated ma terial from the agar is cumbersome.

Paper is a much more suitable supporting material both for analytica and preparative work. The chief problems in paper electrophoresis lie i avoiding the siphoning of buffer down the paper from the electrode vessels and the evaporation of water from the paper due to heating during th run. Both of these prevent the maintenance of a uniform concentration o buffer along the paper. Two types of apparatus originally designed for th separation of amino acids have been used with nucleic acid derivatives One is the apparatus described by Durrum[15] in which the paper strip passes over a glass support and both ends dip into buffer solution, the whole being enclosed in a glass vessel to minimize evaporation. In the second method the paper is held between two glass plates which are cooled by circulating liquid.[16] Neither of these methods satisfactorily overcomes the problem o evaporation from the paper, and both have to be used with comparatively low voltage gradients.

A more convenient technique has been described by Markham and Smith[9, 17] where the paper is kept cool by immersion in carbon tetrachloride. The apparatus (Figure 3) consists of three rectangular glass jars *A*, *B*, and *C* (convenient sizes for most work are *A*, and *B*, 15 x 10 x 5 cm., *C*, 21 x 12 x 6.5 cm.). *A* and *B* contain the buffer solution and *C* contains carbon tetrachloride. A strip of Whatman No. 3 mm. paper, 56 x 8 cm., is soaked in the buffer solution, blotted to remove surplus moisture, and about 0.1–0.2 ml. of a solution containing the mixture to be separated applied in a line across the paper at a suitable place, usually about 12 cm. from one end. The paper is placed in the apparatus so that the two ends dip into vessels *A* and *B* and the center part of the paper is immersed in the carbon tetrachloride in vessel *C*, being held in place by a piece of celluloid or glass (celluloid often contains ultraviolet-absorbing substances soluble in CCl_4). The electrodes, two arc carbons, are placed in *A* and *B* and the whole covered by a glass plate. The electrodes are connected to a d.c. power supply, usually of 1000 v. thus giving a voltage gradient of about 20 v./cm. (Suitable power supplies are shown in Figure 4.) Under these conditions a 2-hr. run is adequate for most separations. The sub-

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[15] E. L. Durrum, *J. Am. Chem. Soc.* **72,** 2943 (1950).

[16] T. Wieland and E. Fischer, *Naturwissenchaften* **35,** 29 (1948).

[17] R. Markham and J. D. Smith, *Biochem. J.* **52,** 552 (1952).

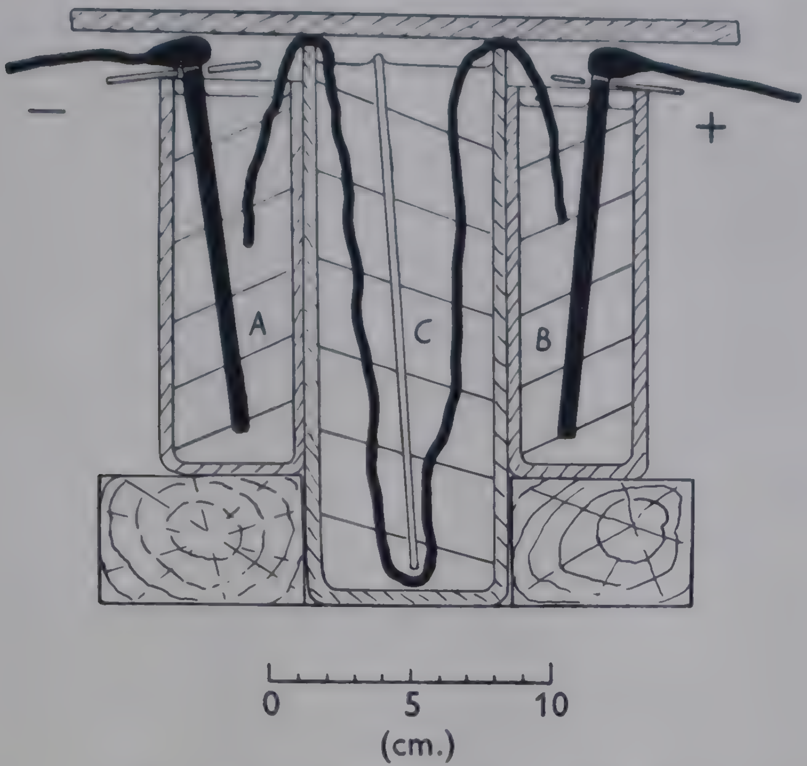

FIG. 3. The electrophoresis apparatus.[17]

stances are located by making a contact print in monochromatic ultraviolet light of 260 mμ,[18, 19] and may be observed during the run by the technique of Holiday and Johnson.[20]

The carbon tetrachloride keeps the paper cool and prevents evaporation. It was chosen for three important reasons: (1) it is reasonably transparent to ultraviolet light; (2) it is nonpolar and so has no tendency to dissolve out the substances from the paper; (3) it is denser than water and so prevents buffer from siphoning over from the electrode vessels and waterlogging the paper.

With Whatman No. 3 mm. paper about 2 mg. substances may be easily run per centimeter width of paper; the only effect of increasing the amounts put on is to make the separating bands broader. Placing the substances in a line rather than in circular spots gives a much sharper separation.

After an electrophoretic run the substances often have to be submitted to further chromatographic analyses. This is greatly facilitated if the buffer components move as spots on the chromatograms, or if they are volatile; for this reason buffers such as ammonium formate and ammonium acetate are the most suitable. Using 0.05 *M* ammonium formate or acetate buffer in the size of apparatus described above, at 20 v./cm. the current is about 8–10 ma. However, with the 0.05 *M* phosphate and borate buffers used for the ranges pH 6–8 and 8–10, it is often necessary to reduce the voltage gradient to about 15 v./cm. to prevent overheating.

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[18] R. Markham and J. D. Smith, *Biochem. J.* **45,** 294 (1949).

[19] R. Markham and J. D. Smith, *Biochem. J.* **49,** 401 (1951).

[20] E. R. Holiday and E. A. Johnson, *Nature* **163,** 216 (1949).

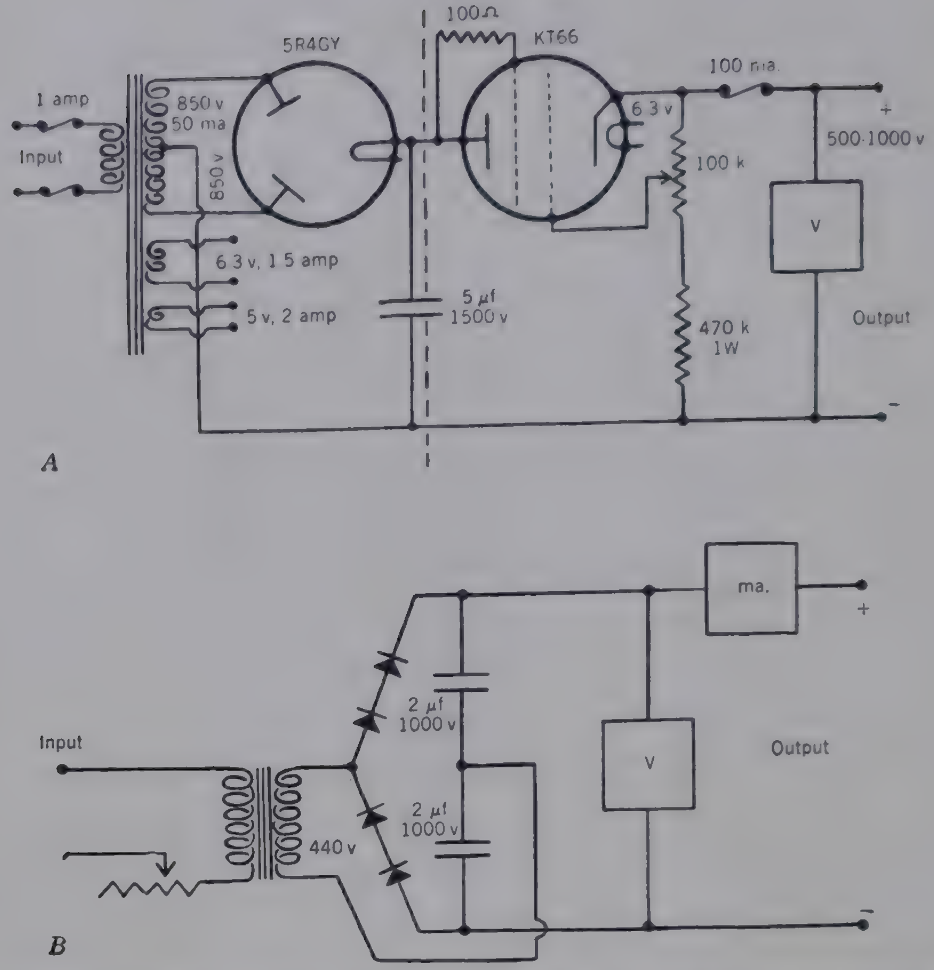

FIG. 4. Two alternative 1000-v. power supplies for the electrophoresis apparatu In circuit B the transformer output must be at least 440 v. r.m.s. at 60 ma. The met rectifiers are in pairs of 350 v. 60 ma. The series resistance in the transformer prima circuit is 1000–5000 ohms rated at 200 ma. and may be replaced with advantage by variable autotransformer.

Under most conditions the movement due to endosmotic flow is ne ligible, but when the substances are placed near one end of the paper ther is an additional movement towards the opposite electrode which appea to be largely due to the movement of buffer into the incompletely saturate paper. In practice this is unimportant, but may be minimized by placir the substances near the center of the paper.

III. Applications of the Method

1. DETERMINATION OF NUCLEIC ACID COMPOSITION

The ease with which adenylic, guanylic, cytidylic, and uridylic aci may be separated by paper electrophoresis leads to a simple method f the determination of the quantitative purine and pyrimidine compositic of pentosenucleic acids (PNA), which has been developed by Davidsc *et al.*[7, 8, 21] The PNA (about 5 mg./ml.) is hydrolyzed to nucleotides

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[21] J. N. Davidson and R. M. S. Smellie, *Biochem. J.* **52**, 594 (1952).

.3 N KOH for 18 hr. at 37°, the solution brought to pH 1 with 60 % wt./wt. HClO$_4$, insoluble KClO$_4$ removed by centrifugation, and the combined vashings and supernatant fluid brought to pH 4. Aliquots of this solution ontaining about 800 μg. of ribonucleotides are run at pH 3.5 in 0.02 M itrate buffer on No. 3 mm. paper (using an apparatus similar to that lescribed by Durrum[15]) for 18 hr. at 11 v./cm. The size of the paper strip s 72 x 7 cm. (Actually by using the technique of electrophoresis on paper mmersed in CCl$_4$[9, 17] and with a shorter length of paper and correspond-ngly higher voltage gradient this separation could be achieved in 30 min-ites.) Whatman No. 1 paper may also be used for smaller quantities of naterial (200 μg.).[8] The nucleotides are located in ultraviolet light at 260 mμ, either visually[20] or photographically,[18, 19] eluted, and estimated spectrophotometrically and by phosphorus determinations. The phosphorus recovery from runs of individual nucleotides is 96–99 %. The PNA phosphorus recovered after alkaline hydrolysis and precipitation of KClO$_4$ is about 97 %, while that recovered as nucleotide P after electrophoresis is 94 % of that in the original PNA. Some of this 6 % loss is probably accounted for by losses during the manipulations, but part has been shown to be due to the presence of small amounts of non-nucleotide P in the PNA specimens.

A similar method may be used for the determination of the composition of deoxyribonucleic acids (DNA) by the electrophoretic separation of the mononucleotides produced on successive hydrolysis with deoxyribonuclease and purified snake venom diesterase. Deoxycytidylic and deoxy-5-methylcytidylic acids, however, are not separated in the electrophoretic run.

Matthews[22] has used paper electrophoresis in borate buffer pH 9 to separate 8-azaguanylic acid from alkaline hydrolysates of the PNA of tobacco mosaic virus isolated from plants treated with 8-azaguanine. The pK of the enol (OH) group of 8-azaguanine is about 3 pH units lower than that of guanine, so that at pH 9·8-azaguanylic acid has a higher mobility than any other mononucleotide.

2. Separation of Nucleotides from Other Phosphorus Compounds

A considerable part of the work on the incorporation of the radioactive isotope P^{32} into PNA has been based on the assumption that the ribonucleotide fraction obtained by the Schmidt and Thannhauser procedure[23] contains only PNA phosphorus.

By separating this ribonucleotide fraction through paper electrophoresis Davidson and Smellie[7] have shown that only about 75 % of the P can be accounted for as nucleotides, there being present at least six additional P

[22] R. E. F. Matthews, *Nature* **171,** 1065 (1953).

[23] G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.* **161,** 83 (1945).

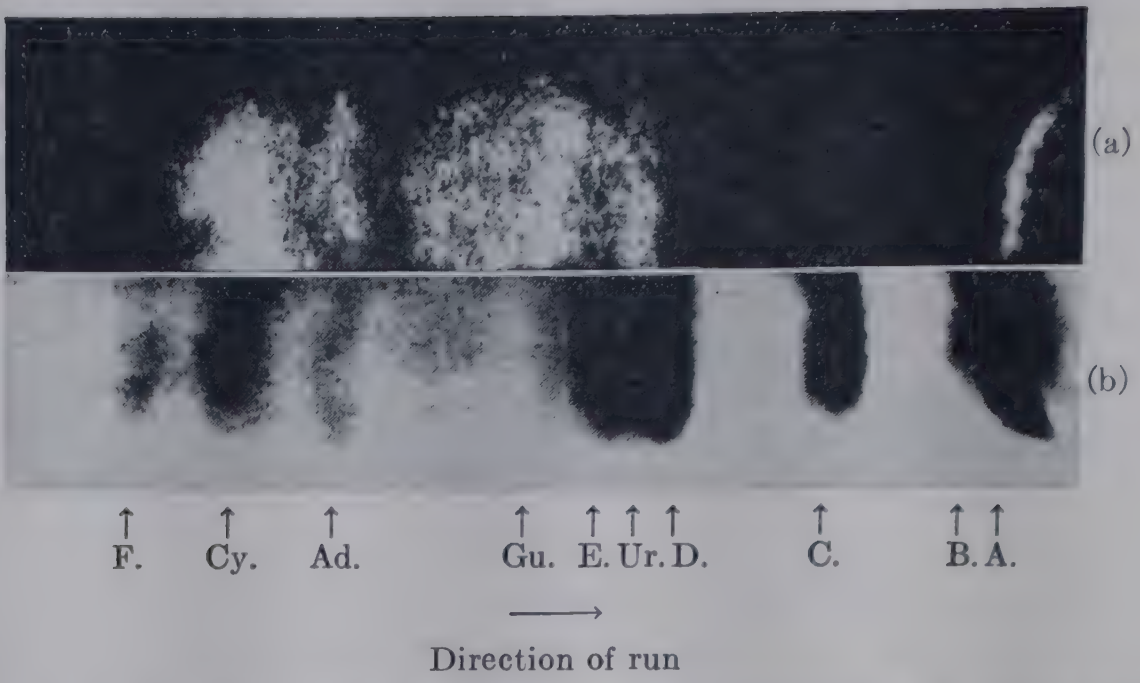

FIG. 5. (*a*) Ultraviolet print at 260 mμ and (*b*) autoradiograph of a short elect phoretic run of rat liver ribonucleotide fraction prepared by a modified Schmidt a Thannhauser method. The substances showing in the autoradiograph contain I Cy, Ad, Gu, and Ur represent cytidylic, adenylic, guanylic, and uridylic acids, spectively. (Reproduced from Davidson and Smellie.[7])

compounds. [Cf. *Smellie*, Chapter 26.] P^{32} was administered subcutaneous to rats and after suitable intervals the animals sacrificed and their liv tissue treated with liquid solvents and cold trichloracetic acid to remo lipid and acid-soluble P. The tissue was then hydrolyzed in 0.3 *N* KO for 18 hr. at 37°, 60% wt./wt. $HClO_4$ added to give pH 1, thus precipitati the DNA and $KClO_4$, the combined supernatant and washings adjusted pH 4 and run on paper electrophoresis at pH 3.5 in 0.02 *M* citrate buffe The nucleotides were located by their ultraviolet absorption and P^{32}-co taining compounds shown up on autoradiographs. The autoradiograp (Figure 5) show the presence of six P^{32}-containing compounds other tha adenylic, guanylic, cytidylic, and uridylic acids, of which one (A) absor in the ultraviolet at 260 mμ. Component B is inorganic phosphate; t other substances are unidentified organic phosporus compounds. As I (administered as inorganic phosphate) is initially incorporated into the substances much more rapidly than into the ribonucleotides, contaminati of the latter by any of these substances leads to gross errors in the est mation of P^{32} incorporation into ribonucleic acid. By a long electrophoret run (18 hr., at 11 v./cm.) cytidylic, adenylic, and guanylic acids may freed from other P^{32}-containing substances and their true specific acti ities measured, but it is difficult to separate uridylic acid from componen D and E. These interfering substances can only be satisfactorily eliminat by the prior isolation of PNA from the tissue.

Deimel and Maurer[24] have described an electrophoretic method for the separation of PNA and DNA fractions which is based on the Schmidt and Thannhauser procedure.[23] After hydrolysis in KOH the solution containing ribonucleotides and DNA is run on paper electrophoresis at pH 8.6, when the ribonucleotides move together and more slowly than DNA. Inorganic phosphate runs just ahead of the DNA. The extent to which these fractions may be contaminated with other phosphorus compounds is not clear.

3. Isolation of Nucleotides and Polynucleotides from Enzymic or Partial Chemical Hydrolysates of Nucleic Acids

The paper electrophoretic technique is perhaps shown to best advantage in the isolation of the products of the partial breakdown of nucleic acids by enzymic or chemical hydrolysis.

A mixture of polynucleotides such as that found in ribonuclease digests contains 30 or more different substances whose direct separation by electrophoresis would be impossible. In practice complex mixtures of this type are first fractionated by running chromatographically on Whatman No. 3 mm. paper in 70% isopropanol–water (vol./vol.) with NH_3 in the vapor phase (solvent 1). This separates the substances produced in the incomplete ribonuclease digestion of PNA into at least 6 bands (which for convenience are numbered 1 to 6 in order of increasing R_F value).[9, 10, 17] Each of these bands contains a relatively small number of substances, many of which may be isolated by a single electrophoretic run.

a. *Cyclic Ribonucleotides*

The cyclic ribonucleotides (nucleoside–2′,3′-monohydrogen phosphates) are intermediates in the acid and alkali breakdown of PNA [Cf. *Brown and Todd*, Chapter 12, and *Baddiley*, Chapter 4] and may be isolated in small yields from very weak alkaline hydrolysates of PNA[17] (e.g., 1 hr. at 100° in water over solid $BaCO_3$). During the course of ribonuclease digestion the cyclic pyrimidine nucleotides are formed as intermediates in the liberation of the pyrimidine mononucleotides,[9, 17] while complete ribonuclease digests contain very small amounts of cyclic purine nucleotides, originating from end groups of the nucleic acid.[25]

As they lack a secondary phosphate group, the cyclic nucleotides move faster than polynucleotides or other mononucleotides on chromatograms run in solvent 1. G! moves just below the cyclic dinucleotides such as AC!, etc. (band 5a), and A!, C!, and U! move together with a higher R_F value (band 6). (The symbol ! is here used to denote a 2′,3′-monohydrogen phosphate group.) These bands to some extent overlap with those of the

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[24] M. Deimel and W. Maurer, *Naturwissenschaften* **39,** 489 (1952).

[25] R. Markham and J. D. Smith, *Biochem. J.* **52,** 565 (1952).

corresponding nucleosides produced during the $BaCO_3$ hydrolysis. Af
elution from the bands the cyclic nucleotides are isolated by electrophore
at pH 3.5, when they run in the positions of the corresponding noncy
nucleotides.

As they bear no secondary phosphate group, at pH 7.4 the cyclic nucl
tides carry only one negative charge, while other nucleotides carry t
(Figure 1). This gives a simple electrophoretic method of separating cy
nucleotides from other mononucleotides.

b. Polynucleotides from Ribonucleic Acid

With the exception of the very small amounts of polynucleotides or
inating from end groups the polynucleotides in complete ribonuclea
digests are of one type.[9, 10, 25] They consist of chains of one or more puri
nucleoside residues joined through 3′,5′-phosphodiester links, and t
minated by a pyrimidine nucleoside residue which is joined through
phosphodiester link on its 5′(OH)- to the 3′- position on the adjacent puri
nucleoside residue, and which bears a singly esterified phosphate in t
3′-position. These are thus of the type, AC, AU, AGC, etc. In partial
gests of PNA are also found the corresponding polynucleotides beari
cyclic (2′,3′-monohydrogen phosphate) groups on the terminal pyrimidi
nucleoside residue, AC!, AGC!, etc. The partial digests also contain sm
amounts of pyrimidine polynucleotides with cyclic end groups such
UU!, UUU!, CC!, etc.

The movement of these substances on a paper chromatogram in solve
1 largely depends on three factors, namely, (1) mononucleotides mo
faster than the corresponding dinucleotides, and dinucleotides move fast
than trinucleotides; (2) substances with cyclic terminal phosphate grou
move faster than the corresponding substances with singly esterified te
minal phosphates; (3) guanylic acid and its derivatives move more slow
than the corresponging mono- and polynucleotides. Chromatography
this solvent thus fractionates ribonuclease digests into a number of ban
from which many individual polynucleotides may be separated by sing
electrophoretic runs at pH 3.5. The chromatographic separation and electr
phoretic mobilities of these are illustrated in Table II. Often more tha
one electrophoretic run is necessary to separate the substances in a sing
chromatographic band. For example band 1 of such a chromatogram elut
and subjected to electrophoresis at pH 3.5 for 2 hr. at 20 v./cm. gives
band moving 13.5 cm. (towards the anode). This is a mixture of GC a
AAC which may be resolved by eluting and either (1) running at pH
when the trinucleotide moves faster than the dinucleotide, or (2) runni
at pH 2.5 for 3 hr. at 16 v./cm., when GC moves 4.8 cm. and AAC 6.4 c

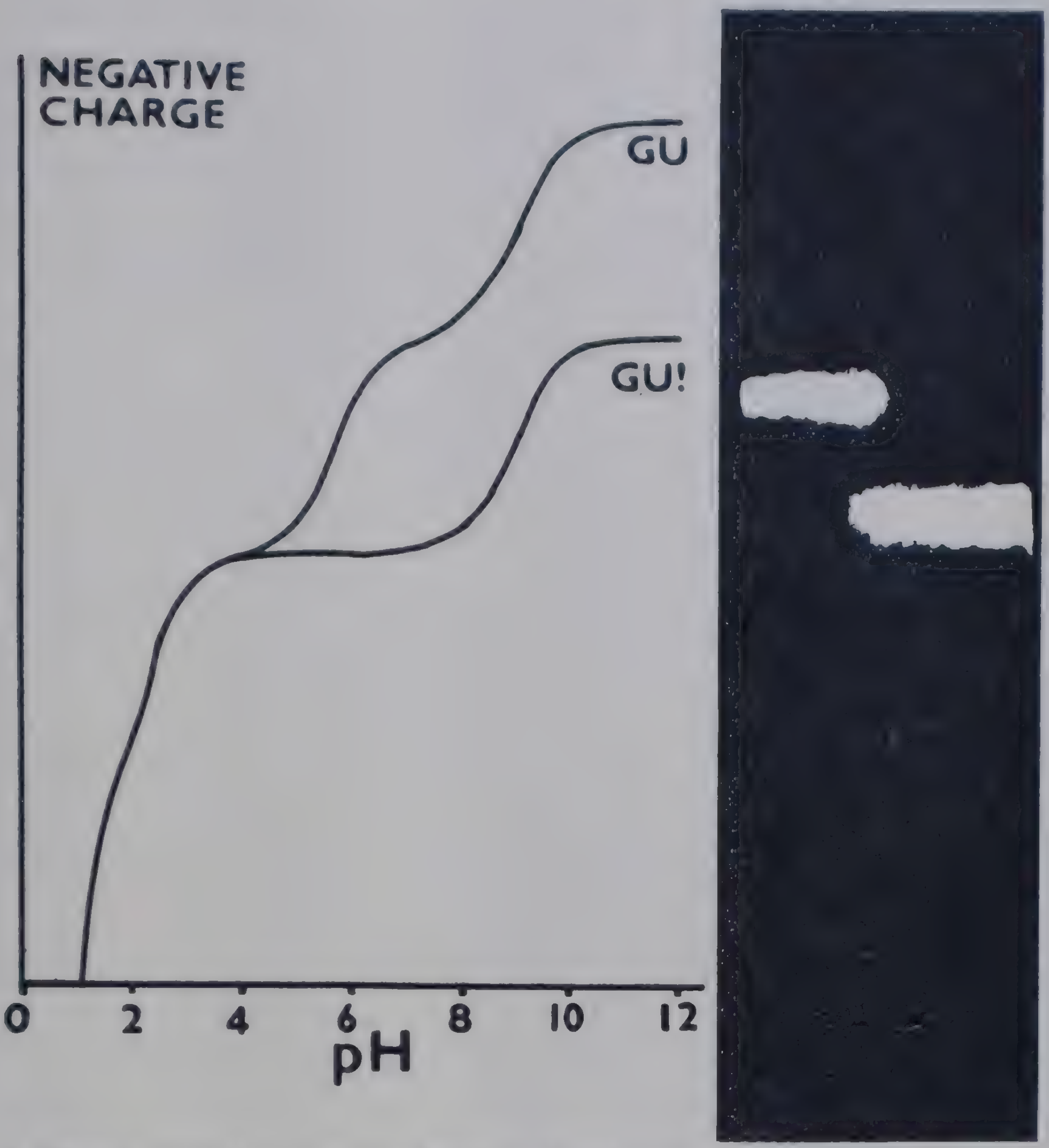

FIG. 6. The dissociation curves of GU and GU! and the electrophoresis at pH 7.4 of GU! and GU (produced by the ribonuclease treatment of GU!).[10] GU differs from GU! in having an extra (secondary phosphoric OH) acidic group. The substances were placed at the top of the paper, GU! on the left and GU on the right.

The dinucleotides with cyclic terminal phosphate groups are separated from the corresponding nucleotides with singly esterified terminal phosphates on the initial chromatogram. These are also separated on electrophoresis at pH 7.4 when the noncyclic dinucleotide bears an additional charge due to the dissociated secondary phosphate group (Figure 6). The polynucleotides in incomplete acid hydrolysates of PNA may be separated in the same way. Although polynucleotides bearing cyclic phosphate groups are absent, these hydrolysates also contain polynucleotides with terminal purine nucleotide residues; consequently, more than one electrophoretic run is often necessary to obtain individual polynucleotides.

Isomers such as AGU, GAU, etc. are not separated on electrophoresis.

TABLE III

The Observed Electrophoretic Mobilities of Some Dinucleoside Monophosphate Diesters and Similar Substances at pH 3.5 in 0.05 M Ammonium Formate Buffer[26, 27]

Substance[a]	Observed movement in 0.05 M ammonium formate buffer pH 3.5 (cm./2 hr. at 20 v./cm.)
A-p-A	4.2
A-p-G	6.0
A-p-C	2.0
A-p-T	6.2
G-p-C	3.0
G-p-MC	3.0
G-p-T	9.0
C-p-C	0
C-p-T	3.9
T-p-T	10.9
A-p-C-p-T	6.2
A-p-MC-p-T	6.2

[a] A, G, C, MC, and T represent deoxyadenosine, deoxyguanosine, deoxycytidine, deoxy-5-methylcytid and thymidine, respectively, and p represents a phosphate linking two nucleoside residues.

c. Polynucleotides and Dinucleoside Monophosphates from Deoxyribonucl Acid

Very similar techniques have been employed to isolate some of the pol nucleotides produced by the action of deoxyribonuclease on DNA.[26] combination of chromatography in solvent 1 and electrophoresis at p 3.5 has been used to separate the dinucleoside monophosphates and anal gous compounds found in deoxyribonuclease digests which have bee treated with phosphomonoesterase.[26, 27] The principles of the electroph retic separation of these substances are similar to those for the separatio of polynucleotides. They contain one less primary and secondary dissoc ation per molecule than do the corresponding polynucleotides, and the effective frictional resistances to motion are slightly different. The electr phoretic properties of some of these are given in Table III.

d. Nucleoside Diphosphates

The nucleoside diphosphates bear two primary and two secondary pho phate dissociating groups. At pH 7 and above they are the fastest-movir nucleic acid components, while at pH 3.5 they are readily separated fro each other and from the corresponding mononucleotides as they bear a additional negative charge due to the second primary phosphate grou

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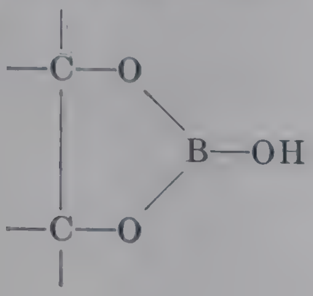
[26] J. D. Smith and R. Markham, *Nature* **170,** 120 (1952).

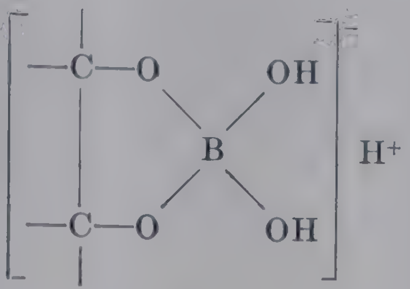
[27] J. D. Smith and R. Markham, *Biochim. et Biophys. Acta* **8,** 350 (1952).

4. SEPARATION OF NUCLEOSIDES AND NUCLEOTIDES AS BORATE COMPLEXES

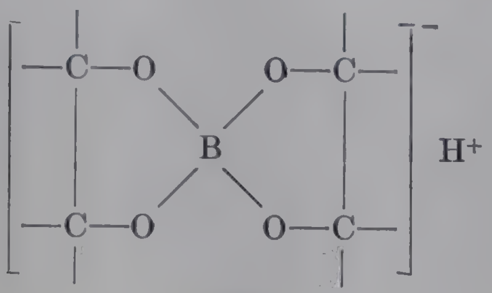
Under certain conditions sugars will form complexes with boric acid believed to be of the types I, II, and III.



I



II



III

Types II and III are only formed when two adjacent *cis*-hydroxyls are present in the sugar ring, and, in dilute solutions, when the relative proportions of borate to sugar are high, type II predominates.[28] It is thus possible by borate complex formation to introduce a new dissociating acid group into sugar residues possessing two *cis*-hydroxyl groups.

The nucleic acid components bearing *cis*-(OH) groups are ribose (which in the pyranose form has four), and ribonucleosides, ribonucleoside-5′-phosphates, and dinucleoside monophosphates from PNA (in the 2′- and 3′-positions). Jaenicke and Vollbrechtshausen[29] have made use of borate complex formation in the separation of some nucleosides and nucleotides by electrophoresis in 0.1 *M* borate buffer pH 9.2. The p*K* of the first dissociation of boric acid is 9.0, so that at this pH the acidic group of the complex is partially dissociated and the ribonucleoside-5′-phosphates bear an additional net charge and, therefore, move faster than other nucleotides. Under these conditions the ribonucleosides move towards the anode in the order (of increasing mobility): adenosine, guanosine and cytidine, uridine; the rate of movement presumably being determined by the negative charges due to the borate complex and those on the enol (OH) groups of the bases. The deoxyribonucleosides which only carry charges on the partially dissociated enol (OH) groups move more slowly than the ribonucleosides.

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[28] H. S. Isbell, J. F. Brewster, N. B. Holt, and H. L. Frush, *J. Research Natl. Bur. Standards* **40,** 129 (1948).

[29] L. Jaenicke and I. Vollbrechtshausen, *Naturwissenschaften* **39,** 86 (1952).

At pH 9.2 in borate buffer the bases, the ribonucleosides, and the nucle tides may be separated into three groups in that order.[14]

Addendum

Microtechnique of Electrophoresis

The electrophoretic techniques hitherto described are suitable for th separation and analysis of the nucleotides or bases from 100 μg. or more o nucleic acid. Edström[30] has developed a method which permits the analysi of 100–1000 pg. of ribonucleic acid (1 pg. = 10^{-12} g.), which is of the sam order as the amount contained in single nerve cells.

The supporting medium is a piece of copper–silk (cellulose) previousl treated with alkali to swell the fibers. The fiber is soaked in a highly viscou citrate buffer pH 3.6, containing glucose and glycerol, and stretched on a quartz slide between dabs of paste moistened with the buffer. During the electrophoresis the fiber is immersed in liquid paraffin. The substances to be separated are placed on the fiber with a micropipet and a potential applied through platinum electrodes between the dabs of paste. With a voltage gradient of 12 v./mm. the adenine, guanine, and cytidylic and uridylic acids from a hydrolysate of PNA in *N* HCl are separated in 2 hr. over a distance of 1 mm. The fiber is photographed together with a light calibration system in monochromatic light of 257 mμ and 275 mμ, respectively, and the densities of the calibration system and parts of the fiber are measured photometrically on a photographic strip.

Detection of Terminal 5′-Phosphate Groups in Ribonucleic Acids

Markham, Matthews, and Smith[31] have shown that the ribonucleic acids of tobacco mosaic virus and potato virus X contain a type of chain which is terminated at one end by a nucleoside residue bearing a singly esterified phosphate group on the 5′-position and at the other end by a nucleoside residue with no singly esterified phosphate group. On alkaline hydrolysis this type of chain gives in addition to mononucleotides, nucleoside-2′,5′- and nucleoside-3′,5′-phosphates from the terminal residues bearing the phosphate groups, and nucleosides from the other end of the chain. These can be separated by paper electrophoresis at pH 7.4 when the nucleosides, which are uncharged, remain at the starting point while the nucleoside diphosphates which carry four negative charges move towards the anode ahead of the nucleoside monophosphates.

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[30] J. E. Edström, *Nature* **172**, 809 (1953).

[31] R. Markham, R. E. F. Matthews, and J. D. Smith, *Nature* **173**, 537 (1954).

CHAPTER 9

Color Reactions of Nucleic Acid Components

ZACHARIAS DISCHE

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The determination of nucleic acids can be based on the determination of one of their three main components, namely phosphoric acid, the sugar, or the nitrogenous bases. As regards the determination of phosphoric acid, this procedure utilizes the differences in alkali lability of the two types of nucleic acid and differences in their solubility in acids. [Cf. *Leslie*, Chapter 16.] It is clear that these methods of determination cannot be regarded as specific, and, while it was found that in certain tissues like liver,[1] the methods give satisfactory results as far as agreement with other methods is concerned, it was reported more recently[2] that in some tissues, like certain tumors, very large discrepancies are encountered between determinatio based on phosphorus and those based on other constituents. Recent several sensitive color reactions for the purine and pyrimidine bases w developed. These reactions have not yet been sufficiently used and test as to their suitability for the determination of the bases in nucleic aci and tissues. More widely used and better tested are methods using col reactions of the sugars of nucleic acids. Because of the fundamental d ference in the nature of the respective sugars in the ribo- and deoxyrib nucleic acids, the methods based on characteristic color reactions of the sugars do not require any separation of the two types of nucleic acids, ar are therefore less tedious and simpler in operation.

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[1] W. C. Schneider, *J. Biol. Chem.* **161**, 293 (1945); **164**, 797 (1946).

[2] L. Drasher, *Science* **118**, 181 (1953).

I. Color Reactions of Nucleic Acids and their Constituents Based on Reactions of Their Sugars

Of the two types of nucleic acid, deoxyribonucleic acid contains in a its nucleotides 2-deoxyribose in the furanoid configuration, while the nucleo tides of the pentose nucleic acids were shown to be ribofuranosides. [C Chapters 2 and 4.] The color reactions of the two types of nucleic aci based on the sugar components are therefore general reactions of the 2 deoxypentoses or pentoses, respectively.

1. Color Reactions of Deoxyribonucleic Acid and Its Nucleotide

Among the color reactions of 2-deoxypentoses so far described thre groups can be distinguished. One represents a type of reaction characte istic for aldehydes and is due to the fact that 2-deoxypentose, in contrast t other saccharides, is present in solution largely in its aldehydic form.[3] second group is represented by a type of reaction given with varying in tensity by various hydroxy and keto aldehydes; and a third by reactions o furan derivatives which are formed from saccharides. It seems noteworth in this connection that the ring form of 2-deoxypentose can, by simpl dehydration, produce furfuryl alcohol, while the straight chain form of th sugar by a simple dismutation between carbon 3 and 4 can be transforme to a highly reactive compound, the ω-hydroxylævulic aldehyde. On th other hand, the transformation of pentoses to the corresponding fura derivatives is probably preceded by an inner rearrangement of the molecul consisting of a dehydration and double bond formation between carbons and 3,[4] and proceeds, therefore, less easily than the formation of fura derivatives of 2-deoxypentose.

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[3] N. G. Overend, *J. Chem. Soc.* **1950,** 2769.

[4] M. L. Wolfrom, R. D. Schuetz, and L. F. Cavalieri, *J. Am. Chem. Soc.* **70,** 51 (1948).

a. Reaction with Diphenylamine

This reaction was first described in 1930[5] and recommended for quantitative determinations of DNA in animal tissues by colorimetry in white light. In 1940 Seibert[6] adapted this method for quantitative determination with a photoelectric colorimeter. The sensitivity of the reaction can be still considerably increased by using a photoelectric spectrophotometer.

Procedure:—The procedure generally used for determinations of DNA is as follows. One volume of the solution containing 50 to 500 μg. DNA per cc. is mixed with a double volume of a reagent, which is prepared by dissolving 1 g. of diphenylamine (twice recrystallized from 70% ethanol or petroleum ether) in 100 cc. of glacial acetic acid of highest purity. 2.75 cc. of concentrated sulfuric acid (reagent grade) is added to 100 cc. of the diphenylamine solution. The reaction mixture is heated at 100° for at least 10 minutes. A blank containing water instead of the unknown is run simultaneously. The DNA solution gives a blue color which does not change significantly for hours. The absorption curve determined with the Beckman spectrophotometer shows a sharp maximum at 595 mμ.

Influence of time of heating and concentration of the acid on the reaction:—Most authors report that the color intensity reaches its maximum after 10 minutes' heating. The color, however, develops very fast in the beginning, so that for qualitative purposes a shorter heating of 3 minutes appears reasonable. Higher concentration of the acid may increase the intensity of the color, but at the same time it decreases the specificity.

Specificity of the reaction:—The blue color developed in the diphenylamine reaction was shown to be produced not only by DNA and 2-deoxyribose, but also by 2-deoxyxylose;[7] it is therefore a reaction of 2-deoxypentoses and not only of 2-deoxyribose. Of the constituent mononucleotides of DNA, the two purine nucleotides give the reaction with an intensity twice that of the equivalent amount of DNA.[8] Preparations of apurinic acid, prepared and analyzed in Dr. Chargaff's laboratory,[9] showed a 4 to 10% lower absorption at 595 mμ than equivalent amounts of DNA. [Cf. *Chargaff*, Chapter 10.] This difference appeared already after 3 minutes' heating and did not increase on further heating. It can be assumed that it is due to a slight destruction of the sugar of the purine nucleotide during the preparation of the apurinic acid. Deriaz *et al.*[10] report that a DNA preparation from fish sperm showed a slightly lower color intensity measured with the Spekker absorptiometer than one-half of the equivalent amount of 2-deoxyribose. Of the pyrimidine nucleotides, thymidylic acid in higher concentration shows a faint blue color and an absorption maximum at 595 m The extinction coefficient of this nucleotide, however, is less than on sixtieth of that of DNA, while cytidylic acid shows no significant color a all. The reaction of DNA, therefore, represents the reaction of its purin nucleotides only. (Brady and McEvoy-Bowe[10a] reported that it is possibl also to determine the deoxypentose of the pyrimidine nucleotides of DN with the diphenylamine reaction after preceding bromination which make the pyrimidine–sugar link more easily hydrolyzable.) It is obvious that th splitting of the glycosidic linkage of the sugar is necessary for the reactio On the other hand, substitution in position 5 and 3 of the sugar by phosphoric acid apparently does not significantly affect the intensity of th reaction. The blue color with the absorption maximum at 595 mμ is no produced by any of the normal sugars or hexals, hexuronic acid, aliphati and aromatic aldehydes, glycolic aldehyde, trioses, aldo- and ketotetrose nor furfuraldehyde.[7] Arabinal, on the other hand, produces the blue colo with the identical absorption spectrum,[7] and the extinction coefficient as measured in the Spekker absorptiometer, is about one-third higher than that of 2-deoxyribose. Finally, Allerton *et al.*[11] recently reported that 3-deoxyxylose, as well as 2,3-deoxyxylose, produces a blue color with the diphenylamine reagent. The extinction coefficient at the maximum of absorption, however, is for both substances about one-thirtieth of that of 2-deoxyribose, and the color develops much more slowly. No data about the absorption maximum and the form of the absorption curve were reported for these two deoxysugars, and it is therefore not established whether the blue color produced is identical with that derived from 2-deoxypentoses.

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[5] Z. Dische, *Mikrochemie* **8,** 4 (1930).

[6] F. B. Seibert, *J. Biol. Chem.* **133,** 593 (1940).

[7] W. G. Overend, F. Shafizadeh, and M. Stacey, *J. Chem. Soc.* **1950,** 1027.

[8] A. E. Mirsky, *Advances in Enzymol.* **3,** 1 (1943).

[9] C. Tamm, M. E. Hodes, and E. Chargaff, *J. Biol. Chem.* **195,** 49 (1952).

[10] R. E. Deriaz, M. Stacey, E. G. Teece, and L. F. Wiggins, J. Chem. Soc. **1949,** 1222

Mechanism of the reaction:—The mechanism of the diphenylamine reaction was extensively studied by Deriaz *et al.*[10] It was found that ω-hydroxylævulic aldehyde, but not lævulic acid, gives with the reagent a blue color with an absorption spectrum identical or very similar to that derived from 2-deoxyribose. They furthermore found that furfuryl alcohol also gives the same reaction. Further studies showed that it is possible to obtain ω-hydroxylævulic aldehyde by heating with methanolic HCl in larger quantities from furfural alcohol and arabinal than from 2-deoxyribose. In agreement with that, the molar extinction coefficient of the first two compounds in the diphenylamine reaction is considerably higher than of the last one. The formation of ω-hydroxylævulic acid from 2-deoxyribose and arabinal involves an inner dismutation between carbon 3 and 4 in which the hydroxyl of carbon 3 is shifted to carbon 4, while in the case of furfuryl alcohol this shift is brought about simply by hydrolysis of the oxygen ring.

[10a] T. G. Brady and E. McEvoy-Bowe, *Nature* **168,** 299 (1951).

[11] R. Allerton, E. G. Overend, and M. Stacey, *J. Chem. Soc.* **1952,** 213.

The ease with which the hydroxyl carbon 3 is shifted is due to its beta position in relation to the carbonyl group which, as shown by Wolfrom *et al.*[4] for other sugars, facilitates this type of dismutation. The weak positive reaction of 3-deoxyxylose could be explained in a similar way by assuming that a small amount of the corresponding 3-deoxyketose is formed by isomerization between carbons 1 and 2. As can be easily seen in this sugar, the shift of the hydroxyl from carbon 4 (in beta position to the carbonyl group) to carbon 5, easily produces also ω-hydroxylævulic aldehyde. Deriaz *et al.*[10] also isolated from the reaction mixture in the diphenylamine reaction 6 different compounds by chromatography and showed that only one of these compounds, representing apparently the product of a reaction between ω-hydroxylævulic aldehyde and diphenylamine, is responsible for the blue color. This compound represents only 10% of the total reaction products; it is yellow at neutral and alkaline reaction and turns blue at a certain acidity.

Interference from other substances:—Most sugars and their analogues, as well as aliphatic and hydroxy aldehydes, produce various colors with the diphenylamine reagent, different from the color given by 2-deoxypentose. When present in solution these compounds, therefore, can interfere with the qualitative and quantitative determinations of deoxynucleotides. The green colors produced by most normal sugars appear with significant intensity only at such high concentrations that in general they will not be of practical importance. Certain carbohydrates, such as agar and carbohydrate from carragheen moss, which contain anhydrosugars give an intensive green color.[12] Of greater importance as far as interference with the reaction of nucleic acids in concerned, are certain compounds of unknown nature which are widely distributed in animal and plant tissues in combination with proteins,[12] and which give an intensive purple color which resembles very much the color produced by hexals and 2-deoxyhexoses;[13] but, as was found in our laboratory, while in the case of the latter two substances the maximum absorption is at 510 mμ (Beckman spectrophotometer), the substances associated with proteins have maxima around 530 mμ. A similar color with absorption maximum at 560 mμ is produced by lipid-soluble substances found in plants.[14] When present in solution in significant amounts, these substances can severely interfere with the reaction of the nucleic acids. Overend *et al.*[15] reported that histone and protamines as well as certain amino acids like glycine, isoleucine, and glutamic acid increase the speed of the development of the blue color in the diphenylamine reactio even at concentrations corresponding to half of the concentration of DN This effect, however, disappears almost completely when the heating continued until the maximum of the color is reached. Cohen[16] found pr viously that the nucleoprotein from calf thymus and the correspondin nucleic acid, show identical color intensity when solutions of the two su stances contained identical amounts of organic phosphorus.[16a]

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[12] N. W. Pirie, *Brit. J. Exptl. Pathol.* **17,** 269 (1936).

[13] W. Ayala, L. V. Moore, and E. L. Hess, *J. Clin. Invest.* **30,** 781 (1951).

[14] M. Ogur, R. O. Erickson, G. Rosen, K. B. Sax, and C. Holden, *Exptl. Cell Research* **2,** 73 (1951).

[15] W. G. Overend, *J. Chem. Soc.* **1951,** 1484.

Detection of DNA and its constituents by dichromatic readings:—When substance which produce colored products with the diphenylamine reagent are present in solution and obscure the reaction of 2-deoxyribose, it is often, accordingto our experiments, possible to detect the presence of the latter by measuring the optical densit of the unknown at two different wavelengths. When substances which produce a gree color are present, the difference of the density at 595 and 650 mμ will in general b either zero or negative for these substances, while it is positive for 2-deoxyribose. If therefore, the amount of the latter substance is not too high, a positive $D_{595} - D_{65}$ will be obtained even when the blue color is no more clearly recognizable. In the cas of acetaldehyde, which produces a brown color, $D_{595} - D_{650}$ is almost zero[10]. In the case of substances which yield purple or yellow colors, the difference of the densitie at 595 mμ and lower wavelengths may also be used to advantage for the detection o 2-deoxypentose.[17]

Quantitative determination of DNA and its constituents:—D_{595} , or its equivalent with photoelectric instruments using filters, is proportional to the concentration o DNA. In general, however, it can be recommended to use $D_{595} - D_{650}$ which, as was found in our laboratory, is also proportional to the concentration of DNA as a measure of this concentration. Although this difference is about 40% smaller than D_{595} , the agreement between duplicate samples was found to be better than when D_{595} is used as a measure of the concentration. In mixtures of DNA and its breakdown products formed by the liberation of bases, the DNA can be determined by carrying out first the reaction on the tissue extract and then bringing the latter to an alkalinity of about 0.1 to 0.2 *N*, heating for 2 minutes at 100°, and repeating the determination. The free sugar of the breakdown product, as was found in our laboratory, is completely destroyed by this procedure, while DNA is not affected. The difference between the two determinations gives then the amount of DNA. By the use of a spectrophotometer as little as 50 μg. of DNA can be determined fairly accurately. Steele *et al.*[18] described a micromethod which permits the determination of as little as 1.6 μg. of DNA by the diphenylamine reaction.

b. Reactions of 2-deoxyribose, DNA and its nucleotides with cysteine and sulfuric acid

2-Deoxyribose gives two completely different reactions when treated with sulfuric acid in the presence of cysteine, depending upon the concentration of the acid and temperature of the reaction. In one of these reactions concentrated acid is added to the solution and all carbohydrates form reaction products with characteristic absorption spectra in the ultraviolet and the blue part of the spectrum. The absorption curves, however, differ from one class of saccharides to another. This reaction will be designated, therefore, as cysteine reaction of 2-deoxyribose with concentrated H_2SO_4. The second reaction is much more selective and can be regarded as a characteristic color reaction of 2-deoxypentose. In it an H_2SO_4–H_2O mixture containing 75 vol. of H_2SO_4 in 100 cc. of the mixture is added to the sugar solution.

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[16] S. S. Cohen, *J. Biol. Chem.* **167,** 691 (1945).

[16a] H. v. Euler and L. Hahn reported (*Arch. neerl. physiol.* **28,** 398, 1946) that certain proteins increase the intensity of the diphenylamine reaction when present in amounts corresponding to twice to three times that of DNA. G. Bergold (*Z. Naturforsch.* **36,** 406, 1948) could not confirm these results.

[17] E. Racker, *Nature* **167,** 408 (1951).

[18] R. Steele, S. Theodore, and L. Ottolenghi, *J. Biol. Chem.* **177,** 231 (1949).

(1) *Cysteine Reaction of 2-Deoxyribose and DNA with 75 Vol.% H_2SO_4.* This reaction was first described in 1944[19] and recommended for quantitative determination in white light. Later Stumpf[20] modified slightly the procedure and adapted it for spectrophotometric measurements. Every one of these modifications offer certain advantages for special purposes, and both precedures therefore are here described.

Original procedure of Dische:—1 cc. of a solution containing 50 to 500 μg per cc. of DNA is mixed with 0.05 cc. of cysteine hydrochloride and 5 cc. of 75 vol.% sulfuric acid (70 vol. of H_2SO_4 conc. plus 30 vol. of H_2O) under cooling in ice. The reaction mixture is then transferred for 5 minutes into a water bath of 40°C. and then cooled in tap water. A pink color develops which after 15 minutes reaches 90% of its maximum, and the maximum after about 1 hour. The absorption maximum is at 490 mμ. The color is stable for hours.

Modification of Stumpf:—0.05 cc. of cysteine hydrochloride and 5 cc. of 75 vol.% sulfuric acid are added to 0.5 cc. of a solution containing 25 or more μg. per cc. of DNA without cooling. The mixture is allowed to cool off at room temperature. The same pink color with an absorption maximum at 490 mμ develops and reaches its maximum after about 15 minutes.

Specificity of the reaction:—The molar extinction coefficients of 2-deoxyribose, deoxyadenylic and -guanylic acids do not differ significantly in Stumpf's modification.[20] Preparations of apurinic acid, however, which react 10% weaker in the diphenylamine reaction than an equivalent amount of DNA, react 18% weaker in either modification of the cysteine reaction. Thymidylic acid, which shows a negligible reaction with diphenylamine, reacts strongly in the cysteine reaction. The molar extinction coefficient for the free thymidylic acid was found[21] to be 74% of that for the purine nucleotides in the Stumpf modification. In the original form of the reaction, however, the thymidylic acid, according to our own observations, shows a completely different behavior. When the optical density is determined immediately after the heating period, it is less than 20% of that of an equivalent amount of DNA. The intensity of the color, however, increases rapidly and the maximum is reached only after about 6 hours. At this momen the molar extinction coefficient corresponds to about 120% of that of DN Deoxycytidylic acid, on the other hand, does not show any color in th reaction. The reaction of thymidylic acid, however, is only so pronounce when free thymidylic acid is present. Thymidylic acid bound in DNA doe not react at all in the cysteine reaction in its original form, as after th destruction of the purine-bound sugar in the apurinic acid by 0.2 N alkal the remaining pyrimidine nucleotides do not show any appreciable colo even after hours. While the purine nucleosides react in the Stumpf mod ification with the same intensity as the corresponding nucleotides, this i not the case with the pyrimidine nucleosides. While the molar extinctio coefficient of thymidylic acid is reported to be 26% lower than that of th purine nucleotides, these extinction coefficients are identical for thymidin and the purine nucleosides. Cytidine, on the other hand, shows a muc lower color than thymidine and the maximum is reached only after 2 hours at room temperature.[21] It must be furthermore noted that the ab sorption spectrum of the pink color in both modifications of the cysteine reaction is not completely identical in the case of thymidylic acid in DNA insofar as the absorption curve for the first one is somewhat steeper towards the lower wavelengths than that for the second one.

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[19] Z. Dische, *Proc. Soc. Exptl. Biol. Med.* **55,** 217 (1944).

[20] P. Stumpf, *J. Biol. Chem.* **169,** 367 (1947).

[21] L. A. Manson and J. O. Lampen, *J. Biol. Chem.* **191,** 87 (1951).

According to our own experiments, the reason for this difference in the behavior o the free and bound thymidylic acid is due to the fact that the latter in polynucleotide linkage always reacts in the cysteine reaction like thymidine-3′,5′-diphosphate. This difference is particularly striking when the reaction is carried out according to the original procedure with the more dilute acid. While under these circumstances the purine nucleotides give the maximum color already 60 minutes after the termination of heating, it takes 24 hours for thymidylic acid to reach the maximum of color; and thymidine diphosphate, isolated from DNA[21a] or prepared synthetically, requires more than 72 hours to reach the maximum. When the purine-bound sugar of DNA is destroyed by alkali after being previously split from the purine by mild acid hydrolysis, the remaining pyrimidine nucleoside diphosphates react in the original form of the cysteine reaction in such a way that the color developed during the first 48 hours at room temperature corresponds to the amount of thymidine diphosphate present in the DNA preparation. This shows that the cytidine diphosphate does not significantly react, at least during the first 48 hours. With increasing concentrations of thymidine diphosphate the intensity of the color increases disproportionately. Nevertheless, it is possible to determine quantitatively the concentration of thymidine diphosphate in a DNA preparation with the cysteine reaction. To this end, the purine sugar is destroyed by 2 minutes' heating at 100° with N H_2SO_4 then adjusting the neutralized hydrolysate to 0.2 N NaOH and again heating for 2 minutes. The cysteine reaction is carried out on this preparation simultaneously with a series of standards prepared from a DNA of known pyrimidine composition, the concentration of the standards to differ by no more than 10% from each other. The concentration of thymidine diphosphate is then obtained by comparing the optical density of the unknown with that of the standard closest to it. As the latter does not differ by more than 10%, the deviation from proportionality can be neglected.

[21a] The author is greatly indebted to Professor A. R. Todd, University of Cambridge, and to the Biochemical Research Foundation, California, for the preparations of thymidine diphosphate used in these experiments.

Neither aldopentoses or hexoses, nor short-chain sugars, glycolic aldehyde, trioses, erythrose, nor aliphatic aldehydes give any reaction with the cysteine reagent, even in much higher concentrations than used for DNA. The same is true of hexuronic acids, and amino, alpha-keto, and alpha- and beta-hydroxy acids, which occur in significant quantities in animal tissues. Fructose gives a yellow color; 2-deoxyhexoses and hexals a yellow-red, and digitoxose a yellow color with completely different spectra from that produced by 2-deoxyribose. The only compound so far investigated related to saccharides which produces a cysteine reaction product with a characteristic maximum at 490 mμ, is arabinal which, of course, under conditions of the reaction should be partly hydrated to 2-deoxypentose. Even in this case, however, the absorption curve differs considerably from that of DNA insofar as it shows two more maxima—one at 450 and one at 415 mμ. The molar extinction coefficient at 490 mμ is only about one-third of that of DNA. Furthermore, arabinal produces a yellow color with an absorption maximum at 470 mμ with sulfuric acid alone in the absence of cysteine. This maximum at 470 mμ disappears when cysteine is added.

Mechanism of the reaction:—The cysteine reaction resembles the diphenylamine reaction as far as its possible mechanism is concerned, insofar as, according to our observation, furfuryl alcohol which gives the characteristic blue color in the diphenylamine reaction, produces also a compound with the characteristic maximum at 490 mμ in the cysteine reaction. However, while the absorption curve of the furfuryl alcohol in the first reaction was found to be identical with that for 2-deoxyribose or DNA, this is not the case in the cysteine reaction. Here the absorption curve shows again two additional maxima which were found in the reaction of arabinal at 450 mμ and 415 mμ, and these two maxima are still more pronounced in the case of furfuryl alcohol. This compound also gives the yellow color with the absorption maximum at 470 mμ with sulfuric acid alone. The molar extinction coefficient at 490 mμ for furfuryl alcohol in both modifications of the cysteine reaction, is considerably lower than that of DNA and arabinal. These observations indicate that either furfuryl alcohol itself or a heterocyclic derivative of it is an intermediate in the cysteine reaction of 2-deoxyribose. It seems much less probable that ω-hydroxylævulic aldehyde or a similar aliphatic aldehyde should be the intermediate in this reaction because hydroxy aldehydes and keto aldehydes, in general, do not react with the cysteine reagent while they give color reactions of varying intensities with the diphenylamine reaction. Furfural, on the other hand, gives a violet color which differs from the absorption spectrum of the coloration obtained with DNA or furfuryl alcohol. The fact that furfuryl alcohol and arabina produce less color than 2-deoxyribose does not necessarily contradict thi assumption about the mechanism of the reaction, as both these substance enter side reactions which are responsible for the additional two absorption maxima, while the intermediate in the case of 2-deoxyribose could be produced in small quantities during a certain time interval and immediately removed by the action with cysteine without being able to enter into side reactions. This assumption seems to be borne out by the fact that thymidylic acid in the original form of the cysteine reaction produces even a higher color intensity than equivalent amounts of purine nucleotides, as in this case the slow splitting of the glycosidic linkage should still more slow down the formation of the intermediate. In agreement with that, the steeper slope of the absorption curve towards lower wavelengths in the case of thymidylic acid also indicates that in this case the side reactions which produce the peaks at 450 and 415 mμ are still more suppressed than in the case of purine nucleotides.

Quantitative determination of DNA and its constituents by the cysteine reaction with 75 vol.% H_2SO_4:—The optical density at 490 mμ of the reaction mixture is proportional to the concentration of DNA, purine nucleotides, and thymidylic acid in the range between 25 and 500 μg. per cc. of DNA or the equivalent amount of the nucleotides in both modifications of the cysteine reaction. It should be noted that, while the molar extinction coefficient for DNA is lower in the original form of the reaction by about one-third as compared with the Stumpf modification, it is possible to make determinations at lower concentrations of the material as the amount of the unknown is twice that used in the other form. As the pyrimidine nucleotides bound in the DNA do not react at all in the reaction, any preparation of DNA can be used as standard solution for its determination in tissues. In the determination of free thymidylic acid or the pyrimidine nucleosides, however, standards of the substance which is to be determined are preferable. Small amounts of pure proteins and carbohydrates in concentrations which occur in animal tissues will not interfere with the reaction. How far this is also true of plants and bacteria will have to be investigated.

(2) *Reaction of DNA with Cysteine and Concentrated H_2SO_4.*[22]

Procedure:—To 1 cc. of a solution containing 50 to 500 μg. per cc. of DNA, 4 cc. of concentrated sulfuric acid (reagent grade) is added under cooling in tap water. The reaction mixture is left for 1 hour at room temperature to avoid air bubble formation, and then 0.1 cc. of a 3% solution of cysteine hydrochloride is added and shaken. The solution is left from 20 to 48 hours at room temperature, and the absorption is then measured with the Beckman spectrophotometer.

Absorption curve and specificity of the reaction:—Pentoses, hexoses, heptoses, methylpentoses, and hexuronic acids form, under the conditions of this reaction, furfural or the corresponding homologues which all show intensive absorption between 305 and 330 mμ,[22] or at 405 mμ.[23] This absorption appears immediately after the addition of the sulfuric acid to the solution of the sugar. With DNA or its nucleotides, no such absorption appears before addition of cysteine. Subsequently, a compound is slowly formed which shows a sharp absorption maximum at 375 mμ. The maximum absorption is reached at room temperature only after 48 hours, but 80% of the maximum is already obtained after 20 hours. Apurinic acid, thymidylic acid, arabinal, and furfuryl alcohol, according to our more recent observation, give the same reaction and the slope of the absorption curve, between 350 and 380 mμ, is identical in all these cases with that obtained with DNA. The molar extinction coefficients at 375 mμ, however, are considerably decreased, *viz.*, for apurinic acid by 30%, for arabinal by 57%, for furfuryl alcohol 53%, and by 40% for thymidylic acid. These values indicate that the mechanism in this reaction is identical or similar to that in the cysteine reaction of DNA with 75 vol.% H_2SO_4, and that either furfuryl alcohol itself or a furan derivative closely related to it is an intermediate in this reaction. This conclusion is supported by the fact that apurinic acid gives lower values than equivalent amounts of DNA, as it can be assumed that in APA at least a part of the sugar is present as a straight-chain compound. The lower values for furfuryl alcohol do not exclude the possibility that this compound itself is an intermediate, as here again side reactions may occur when the compound is present from the beginning, and may be lessened or altogether voided when it is formed slowly from another compound.

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[22] Z. Dische, *J. Biol. Chem.* **181,** 379 (1949).

Interference from other substances:—As all carbohydrates give absorption spectra in this cysteine reaction, with peaks around 400 mμ, they will all interfere with the determination of DNA. As the reaction product of pentoses, however, is unstable and most of the absorption due to it disappears after 24 hours' standing at room temperature,[22] the interference from small amounts of ribonucleic acid in general will not be significant. As far as the interference from hexoses is concerned, it may be possible to eliminate it by dichromatic readings at 375 mμ, and a higher wave length chosen in such a way that the absorption due to hexoses which has a maximum at 414 mμ, is equal to the absorption at 375 mμ. The difference in the optical density of these two wavelengths will then be a measure of the amount of DNA or one of its breakdown products. On the other hand, aliphatic aldehydes and hydroxy aldehydes such as glycolic aldehyde, trioses, tetroses, and pyruvic aldehyde do not react at all in the general cysteine reaction of carbohydrates and will not interfere with it. The same is true of amino acids and pure proteins in considerable amounts, exceeding several times that of DNA, so that the reaction could be used to determine DN in nucleoproteins.

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[23] Z. Dische, *J. Biol. Chem.* **204,** 983 (1953).

c. *Reaction of DNA with Tryptophan and Perchloric Acid*

This reaction, described by Cohen in 1944,[16] represents a special case o a more general reaction of carbohydrates with tryptophan in acid solutio described by Thomas.[24]

Procedure:—To 1 cc. of a solution containing 100 to 500 μg. of DNA are added 0.2 cc. of 1% tryptophan solution and 1.2 cc. of 60% perchloric acid. The solution i heated in a boiling water bath for 10 minutes, cooled in tap water, and read in th spectrophotometer 5 minutes later. A purple color appears. The absorption spectrum has so far only been determined with a Klett photoelectric colorimeter. The maximum is obtained with a filter having a transmission range between 485 and 550 mμ. According to our own observation, an absorption maximum at 500 mμ is obtained with th Beckman spectrophotometer.

Specificity of the reaction:—Deoxyguanosine was found to react with a molar extinction coefficient identical with that of DNA. This suggests that the pyrimidine nucleotides of DNA react in this reaction to the same extent as the purine nucleotides. This conclusion, however, should be further checked by direct determinations on isolated pyrimidine nucleotides as well as breakdown products in which the purine sugar is destroyed. Aldopentoses, aldo- and ketohexoses, trioses, ascorbic acid, furfural, acetaldehyde, benzaldehyde, glyceraldehyde, and palmitaldehyde give color reactions with the reagent which, however, differ in their absorption maximum when determined with the Klett photoelectric colorimeter. Rhamnose apparently showed the same maximum as DNA, while the intensity of the color corresponded only to 4% of that of an equal amount of DNA.

Mechanism of the reaction:—Cohen[16] suggested that the colored product is a Schiff base produced by the condensation of 2-deoxyribose with the nitrogen of the pyrrol ring. This suggestion is based on the assumption that 2-deoxyribose, differing from other carbohydrates with a hydroxyl at carbon 2, would not be able to participate in the Amadori reaction and thus an intermediate containing conjugated double bonds would accumulate. Observations in our laboratory have shown that furfuryl alcohol reacts with Cohen's reagent producing a pink color with an absorption maximum at 500 mμ. The absorption curve is like that of arabinal and not identical with that of DNA, and the molar extinction coefficient at 500 mμ 40% lower than that for DNA. This and the reactivity of hydroxy aldehydes with the reagent suggest that here, as in the diphenylamine reaction, not the aldehydic form of 2-deoxypentose, but an aldehydic intermediate which can be formed from the sugar as well as from furfuryl alcohol, is responsible for the color reaction.

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[24] P. Thomas, *Z. Physiol. Chem.* **199,** 10 (1931).

Quantitative determination of DNA in the presence of other substances:—The color leveloped in the reaction is proportional to the concentration of DNA in the range of .01 to 0.05% when determined with a Klett photoelectric colorimeter. Aldehydes, ructose and its derivatives can interfere, however, with the reaction. The protein noiety in nucleoproteins interferes by creating a turbidity and producing unspecific olors due to the protein constituents. The first impediment can be eliminated by iltration; the second by extraction with isoamyl alcohol of a boiling point of 130–132°, vhich does not extract the color produced by histone or protamine constituents but xtracts completely the color due to deoxyribose. The standard solution, of course, nust be treated in an identical way. Other proteins, however, may interfere and the xtraction with isoamyl alcohol may be of no avail.

l. Reaction of DNA and 2-deoxyribose with Indole and HCl

This reaction was first described in 1929.[25] As the original procedure shows much lower sensitivity than other reactions, Ceriotti[26] modified it by increasing the acid concentration. This more sensitive modification, however, appears to be less specific.

Original procedure:—To 1 part of a solution containing 0.5 to 2.5 mg. per cc. of DNA, are added an equal volume of 1% HCl and 0.1 cc. of a 1% solution of indole in ethanol. The mixture is heated for 5 minutes in a boiling water bath. An intensive reddish-brown color appears. The reaction mixture is thoroughly shaken with an equal volume of chloroform, whereupon the water phase becomes yellow-brown while reddish solid particles accumulate at the interphase.

Specificity of the reaction:—The reaction is produced only by the purine nucleotides of DNA, as destruction of the sugar of purine nucleotides by protracted heating in 2% sulfuric acid destroys the reactivity of DNA. Pentoses and hexoses even at 2.5%, or ribonucleic acid at 0.25%, do not produce any visible color in the water phase. On the other hand, glucosone and the reaction products obtained by deamination of glucosamine with nitrite (probably 2,5-anhydromannose) give the same yellowish-brown color as DNA.

Procedure of Ceriotti for the quantitative determination of DNA:—To 2 cc. of a solution containing 2.5 to 15 μg. per cc. of DNA, are added 1 cc. of 0.04% indole C.P. solution in distilled water and 1 cc. of concentrated HCl (sp. gr. 1.19). The test tube is immersed for 10 minutes in a boiling water bath and cooled under running water, the solution is extracted 3 times with 4 cc. of chloroform, and the water separated by centrifugation from the chloroform. As the purity of the chloroform is of the utmost importance, it should be purified by repeated extraction with concentrated H_2SO_4, followed by water extraction, and then freed of water by keeping it for 48 hours over $CaCl_2$. Finally, it is distilled to give a product with the boiling point of 61°C. A yellow color appears in the water phase which is read with the Beckman spectrophotometer against a blank treated in an identical manner. A faint pink color appears in the chloroform phase. The color given in the water phase is stable for several hours. On long standing at room temperature, a pink color forms which can be again removed by extraction with chloroform. The absorption curve has a sharp peak at 490 mμ and second small but constant peak at 460 mμ.

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[25] Z. Dische, *Biochem. Z.* **204,** 431 (1929).

[26] A. Ceriotti, *J. Biol. Chem.* **198,** 297 (1952).

Specificity of the reaction according to Ceriotti:—Apurinic as well as thymidylic acid according to our observations produce the same color as DNA the molar extinction coefficient of thymidylic acid, however, is about 20% that of DNA. Free 2-deoxyribose gives a much lower color intensity tha DNA. The same is true of arabinal. Glucosamine, lævulic and uric acids as well as creatine and ascorbic acid, do not give any color. Hexoses, on th other hand, give a pink color which is completely extracted by chloroform Arabinose, and probably also other pentoses as well as RNA, however produce a brown color in the water phase which corresponds to 8% of th color produced by an equivalent amount of DNA. Other pentoses and RN were not investigated but probably behave similarly. Galacturonic aci also produces a considerable yellow-brown color in the water phase. Thes two types of substances, therefore, may interfere with the determinatio of DNA, if present in excess of DNA, and must be accounted for afte determination of their concentration in the unknown.

Mechanism of the reaction:—Not only tryptophan but other β-substitute derivatives of indole give color reactions when heated with $HClO_4$ an DNA. This, and the fact that hydroxy and keto aldehydes produce mor or less intensive colors[16] in both modifications of the indole reaction as well as in the tryptophan reaction, suggests that the first reaction resembles the second one in its mechanism. Furfuryl alcohol, however, does not produce any color in the water phase in the Ceriotti modification, and a very faint one only in the original form of the indole reaction. This, like the weak reaction of free deoxyribose, may be due to a rapid destruction of these compounds by HCl at 100°.

e. *Reaction with Carbazole and Sulfuric Acid.*

This reaction, described in 1930[5] is best carried out according to the modification of Gurin and Hood.[27] DNA shows a purple color with an absorption maximum at 530 mμ. The maximum of the color is already reached after 2 minutes at 100°.

Specificity of the reaction:—A characteristic feature of this reaction is that after destruction of the sugar of the purine nucleotides by heating for 2 hours with 2% H_2SO_4, the remaining pyrimidine polynucleotides react with about the same intensity as the whole DNA molecule, in spite of the fact that the free purine nucleotides also react with great intensity.[28] This indicates a considerable influence of the linkages between the nucleotides on the intensity of the reaction. Free thymidylic acid reacts about 10%

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[27] S. Gurin and D. B. Hood, *J. Biol. Chem.* **139,** 775 (1941).

[28] H. Angermann and F. Bielschowsky, *Z. physiol. Chem.* **191,** 123 (1930).

less than half the equivalent amount of DNA. The reaction is not specific as practically all saccharides give purple colors with slightly different absorption maxima. Aliphatic aldehydes and hydroxy aldehydes also give intensively colored products.

f. Reaction of DNA with Schiff's Reagent

The presence of considerable amounts of the aldehydic form of 2-deoxyribose in its solutions manifests itself by its ability to produce a characteristic red color with Schiff's reagent. Feulgen[29] found in 1924 that after a brief hydrolysis with 1 *N* HCl, DNA reacts with Schiff's reagent. Tobie[30] introduced a modified reagent characterized by a much higher concentration of SO_2 which increases considerably the sensitivity of the reaction. Widström[31] adapted the reaction with Schiff's reagent for quantitative purposes. While the reaction proved of great importance for cyto- and histochemical studies on nucleic acids [Cf. *Swift*, Chapter 17], it cannot compare in sensitivity with the other methods here described, as far as quantitative determinations are concerned.

g. Evaluation of Various Methods of Quantitative Determination of DNA and Its Constituents

Of all the reactions here described, that with cysteine and 75 vol. % sulfuric acid appears to be the most specific, as even a compound as closely related to 2-deoxyribose as arabinal, produces a color which can be distinguished by its absorption spectrum from that of 2-deoxyribose. It will be of particular interest to see whether 3-deoxypentose and 2,3-deoxypentose which, according to Overend,[7] show a weak blue color in the diphenylamine reagent, react at all with the cysteine reagent. For quantitative determination, however, the difference in specificity between this reaction and that with diphenylamine appears negligible and the sensitivity of the latter reaction is about twice that obtained with the modification of Stumpf of the cysteine reaction with 75 vol. % H_2SO_4. The cysteine reaction of DNA, furthermore, may prove valuable for the determination of free thymidylic acid in the presence of other deoxynucleotides or DNA, particularly in its free form. However, more investigations appear necessary to test the application of the cysteine–H_2SO_4 reaction for determinations in living tissues. The tryptophan–perchloric acid reaction, when measured with the Beckman spectrophotometer, appears to have about one-third of the sensitivity of the diphenylamine reaction. Its use for quantitative purposes in living cells has not sufficiently been studied, and the data so far published suggest that the chromogen is more labile than that of the d phenylamine reaction, and that the presence of even small amounts proteins makes the determination more laborious. As in this reaction th pyrimidine nucleotides seem to react as well as purine nucleotides, it ma prove useful for the detection of breakdown products of DNA. The ca bazole reaction could be used for the same purpose. Although the latter i less specific than the tryptophan reaction and is strongly interfered with b glucose, it is less affected by proteins. The reaction with indole and HCl i Ceriotti's modification appears of interest, as it makes possible quantitativ determinations on about 10 times small amounts of material than all th other methods. The use of this method in combination with special micro-techniques like that of Steele *et al.*[18] may make it possible to determin DNA in quantities below 1.6 μg. per. cc. The lesser specificity, however, o this reaction and the lack of sufficient data about its application for the quantitative determination of nucleic acids in tissues and of the effect o proteins on the reaction suggest caution in its use. The availability of several color reactions of 2-deoxypentose which differ in their mechanism, specificity, and in the degree of interference from other substances is of particular advantage, as far as detection and identification of DNA and its derivatives in living cells is concerned. The uncertainties inherent in the use of color reactions for this purpose are largely eliminated by checking, on a quantitative basis, the results of one of these reactions by those obtained from several other ones.[31a]

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[29] R. Feulgen and H. Rosenbeck, *Z. physiol. Chem.* **135,** 203 (1926).

[30] W. C. Tobie, *Ind. Eng. Chem., Anal. Ed.* **15,** 405 (1942).

[31] G. Widström, *Biochem Z.* **199,** 298 (1928).

2. REACTION OF RIBONUCLEIC ACIDS

All reactions of ribonucleic acids based on the sugar component, so far published, are general reactions of pentoses. Three such reactions were tested for quantitative determinations of PNA.

a. Orcinol Reaction

This reaction was recommended in two different forms—one, using $FeCl_3$ as catalyst, by Bial,[32] and one using $CuCl_2$[33] which was employed for the determinations of ribonucleic acids and its nucleotides by Massart and Hoste.[34] The Bial reaction, first used by Embden and Lenhartz[35] for white light colorimetry of free pentose in 1924, was adapted for quantitative determinations and differentiation of various nucleotides by spectrophotometric measurements in 1937.[36] Several modifications[37] of this reaction were later developed which differ in the ratio between the solution and the reagent, and the composition of the latter, as regards the concentration of HCl, $FeCl_3$, and orcinol. The original form of the reaction, as proposed in 1937, uses a higher HCl concentration than all later modifications and is, therefore, the most sensitive of all. The most widely used modification seems to be that proposed by Mejbaum[38] in 1939 which uses 6 N HCl.

[31a] The reaction of DNA with phloroglucinol described by H. v. Euler and L. Hahn (*Arch. neerl. physiol.* **28,** 398, 1946) appears to be 20 times less sensitive than the diphenylamine reaction and does not seem to offer any advantage as compared with other reactions of DNA.

[32] M. Bial, *Deut. med. Wochschr.* **29,** 253; **29,** 477 (1903).

[33] H. Barrenscheen and A. Peham, *Z. physiol. Chem.* **272,** 81 (1942).

[34] L. Massart and J. Hoste, *Biochim. et Biophys. Acta* **1,** 83 (1947).

[35] G. Embden and E. Lenhartz, *Z. physiol. Chem.* **201,** 149 (1931).

Quantitative determination of PNA according to Dische and Schwartz:[36]—To 1.5 cc. of a solution of ribonucleic acid is added 3 cc. of the reagent which is prepared by dissolving 100 mg. $FeCl_3 \cdot 6H_20$ in 100 cc. of HCl, sp. gr. 1.19, and adding 3.5 cc. of a 6% solution of orcinol (twice recrystallized from benzene) in ethanol. The reaction mixture is heated in a water bath for 3 minutes and cooled in tap water. A standard of PNA and a blank containing water instead of the unknown is run simultaneously. The optical density is measured at 665 mμ with the Beckman spectrophotometer against a blank containing water and the reagent. A solution of PNA containing 40 μg. per cc. shows an optical density of 0.18 which does not differ significantly from the density of a solution of 20 μg. per cc. of adenosine-3-phosphate. The optical density is proportional to the concentration of PNA in the range between 10 and 100 μg. per cc.

Quantitative determination of PNA according to Mejbaum:[38]—To 1 part of the unknown is added an equal volume of concentrated HCl, sp. gr. 1.19, containing 0.1% $FeCl_3 \cdot 6H_2O$ and 0.1% of orcinol. The mixture is heated for 20 minutes, or, according to Albaum and Umbreit,[39] for 45 minutes. A standard solution of PNA and a water blank are run simultaneously and the optical density of the solution is measured at the absorption maximum, which with the Beckman spectrophotometer lies at 670 mμ

Specificity of the reaction:—In all the modifications of the Bial reaction only the purine nucleotides and nucleosides react significantly. A more involved procedure for the determination of pyrimidine nucleotides in which bromination and prolonged heating with acid at 105° precedes the performance of the Bial reaction, was developed by Massart and Hoste.[34] Bial's orcinol reaction has a rather low specificity as not only pentoses, but also 2-deoxyribose and DNA, methylpentose, and hexuronic acids give a green color with an absorption maximum around 670 mμ. Certain aldoheptoses which can occur in bacterial polysaccharides also produce a green color with an absorption maximum around 655 mμ.[40] All these substances can occur in tissue extracts as they are prepared for determination of PNA.

Interference from other substances:—Aldo- and ketohexoses in free form and in polysaccharides produce, in all modifications of Bial's orcinol reaction, a reddish-brown color which can severely interfere with the reaction of PNA. Proteins and certain lipidic cell constituents can also interfere b forming red products.

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[36] Z. Dische and K. Schwarz, *Mikrochim. Acta* **2,** 13 (1937).

[37] G. L. Miller, R. H. Golder, and E. E. Miller, *Anal. Chem.* **23,** 903 (1951).

[38] W. Mejbaum, *Z. physiol. Chem.* **258,** 117 (1939).

[39] H. G. Albaum and W. W. Umbreit, *J. Biol. Chem.* **167,** 369 (1947).

[40] Z. Dische, *J. Biol. Chem.* **204,** 983 (1953).

Quantitative determination of PNA in presence of aldohexose:—When the amoun of glucose or its polymers reaches a certain level, the absorption at 670 mμ due to th brown reaction products can increase the total reading. If the amount of glucose i not too high, it is possible to eliminate this discrepancy by dichromatic readings With Mejbaum's reagent, the procedure recommended by Brown[41] and by Drury[4] is used. This consists in the simultaneous determination of pentose and glucose fron two equations obtained from two measurements of optical densities at two differen wavelengths. Using the procedure of Dische and Schwartz, after the determinatio of optical density at 665 mμ, the optical density is determined at the wavelengt around 565 mμ, at which the optical density of a glucose standard run simultaneousl with the unknown shows the same optical density at 665 mμ.[43] The difference of $D_{665} - D_{565}$ of the experimental sample divided by this difference in the internal standard i then a measure of the concentration of PNA. This difference is only about 25% lowe than D_{665} so that this procedure does not involve a large decrease in sensitivity of th reaction. It is not possible, however, to use such a procedure in the presence of ketohexoses or ketoheptoses.

b. *Phloroglucinol reaction of Euler and Hahn*[44]

Procedure:—To 1 cc. of the unknown containing 2 mg. PNA is added 8 cc. of a 0.1% solution of $FeCl_3$ in a mixture of 1 part of concentrated HCl and 6 parts of glacial acetic acid. After stirring, the tubes are immersed for 50 minutes in a boiling water bath and cooled to room temperature. 1 cc. of a 25% phloroglucinol solution in a mixture of 1 part concentrated HCl, 1 part H_2O, and 2 parts of glacial acetic acid is then added and kept for 20 minutes at room temperature. The tubes with the reaction mixture are then immersed in a boiling water bath for exactly 4 minutes, cooled to room temperature, and so kept from 2 to 24 hours. The maximum of the color intensity appears after 10 hours. The absorption maximum is at 680 mμ read with the Beckman spectrophotometer.

Specificity of the reaction:—DNA does not give any color with this procedure; this, however, could be due to the prolonged heating before the addition of phloroglucinol which, of course, destroys the sugar of the purine nucleotides of DNA. No data were reported as to the reactivity of other sugars and aldehydes.

c. *Reaction of Pentose with Cysteine and H_2SO_4*

PNA, its nucleotides and nucleosides all react in the general reaction of carbohydrates with cysteine and sulfuric acid. The procedure was described above under I.1 b. The readings have to be carried out about 15 minutes after addition of cysteine when the maximum absorption produced by pentoses is observed. The peak of the absorption curve is at 390 mμ.

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[41] A. H. Brown, *Arch. Biochem.* **11,** 269 (1946).

[42] H. F. Drury, *Arch. Biochem.* **19,** 455 (1948).

[43] Z. Dische, G. Ehrlich, C. Munoz, and L. von Sallmann, *Am. J. Opthalmol.* **36,** 54 (1953).

[44] H. v. Euler and L. Hahn, *Svensk Kem. Tidskr.* **58,** 251 (1946); *Chem. Abstr.* **41,** 2108 (1947).

Reaction of nucleotides and nucleosides of PNA:—Only the purine nucleotides of PNA react significantly in the cysteine reaction. The molar extinction coefficient of adenosine-3-phosphate does not differ significantly from that of pure ribose, and is twice as great as that of PNA.

Quantitative determination of PNA and its constituents in presence of DNA and hexoses:—The optical density in the cysteine reaction is proportional to the concentration of pentose in the range of concentration between 2 and 40 μg. per cc. of ribose. The density decreases slowly after maximum absorption is reached, and for this reason the readings of the optical density of the unknown must be carried out simultaneously with the readings of the standard solution. The purest preparation of DNA showed an optical density at 390 mμ, which corresponded to no more than 1.5% of that of equivalent amounts of PNA. As this ratio was obtained for several preparations of DNA, it is probably not due to contamination of the prepartion with small amounts of PNA. Aldohexoses produce with the cysteine reaction a yellow color with an absorption maximum at 412 mμ. This color interferes with the determination of the pentose. This interference, however, can be eliminated by dichromatic readings. The latter are carried out by finding, around 424 mμ, a wavelength at which a standard of glucose or another aldohexose shows the same optical density as at 390 mμ. $D_{390} - D_{424}$, therefore, is zero for the hexose. It is only slightly lower than D_{390} for the pentose. In this way adenosine-3-phosphate can be still determined in presence of a 5-fold amount of glucose or a 10-fold amount of mannose or galactose. $D_{390} - D_{424}$, however, is not zero for ketohexoses. Hexuronic acids show an identical absorption curve as pentose, although the molar extinction coefficient is far smaller. When present in large quantities, however, they will interfere with the reaction, and the same is true of methylpentose. It is therefore necessary to correct for the presence of these two classes of saccharides as in the case of the orcinol reaction.

Evaluation of the PNA reactions:—Of the three reactions, that with Bial's reagent appears most sensitive and the only one sufficiently investigated as far as the use for quantitative determinations in tissues is concerned. The procedure according to Dische and Schwarz is more sensitive than the other modifications and therefore allows a shorter heating time. This in turn decreases the interference from other sugars. As regards interference from proteins, the orcinol reaction appears to be more influenced than the cysteine–H_2SO_4 reaction. The latter reaction is also more suitable for the detection of small amounts of PNA in DNA. The reaction with phloroglucinol of Euler and Hahn is much less sensitive, appears more laborious, and has not been sufficiently investigated as far as interference from other substances is concerned.

II. Determination of Purine and Pyrimidine Bases of Nucleic Acids by Characteristic Color Reactions

The discovery that the molar ratios of individual purines and pyrimidines in nucleic acid varies from one preparation of nucleic acid to another depending on the species from which it was obtained [Cf. Chapters 10, 11 rendered the determination of the content of these bases by simple an sensitive reactions a matter of considerable interest. In the last few year such methods were developed for adenine, thymine, cytosine, and uraci which are fairly characteristic for these substances and could perhaps b applied for the analysis of nucleic acids. A micromethod for the deter mination of guanine in urine has not yet been applied to the determinatio of this purine in nucleic acids and will not be discussed here. Unfortunately the preparations used by various authors for testing the applicability o these methods for the determination of the bases in nucleic acids were not completely pure and it is difficult, therefore, at present to evaluate how far these methods could give completely correct values for the content of individual purines and pyrimidines in nucleic acids and how far they can be applied for the determination of these substances in tissue extracts without interference from other cell constituents.

1. Determination of Adenine

The procedure of Woodhouse[45] is based on a color reaction of adenine after its reduction with zinc dust and diazotization with $NaNO_2$ with *N*-1-naphthylethylenediamine hydrochloride. The red color is still visible at a concentration of 5 μg. per cc. of adenine and is proportional to its concentration up to 40 μg. per cc. The determination of adenine in adenosine and adenosine-3-phosphate gave correct values. In two preparations of nucleic acids, the P content of which was about 20% below the theoretical, the values were 10% lower than corresponded to the content in P. The reaction is not specific for adenine, as it was originally proposed for the determination of folic acid.

2. Determination of Thymine

Two procedures by Woodhouse and by Pircio and Cerecedo for the determination of thymine are both based on the Hunter[46] reaction for this substance. Thymine is coupled first with sulfanilic acid in Na_2CO_3 solution, and the reaction product then treated with hydroxylamine in NaOH. A red color is produced which is still visible at a concentration of 10 μg. per cc. of thymine. Cytosine and uracil do not interfere, but purines must be removed either as silver salt or by palladium chloride. Woodhouse[47] precipitates thymine as a silver salt before its determination. Pircio and Cerecedo[48] extract the evaporated hydrolysates of nucleotides or nucleic acids ith ether to remove the lævulic acid produced from the sugar. The reaction can be obtained with solutions containing only 10 μg. per cc. of hymine. The involved procedure for the purification of the base requires nuch higher quantities when thymine is determined in DNA or PNA. The alues for thymine obtained on preparations of nucleic acids are in the range ound by other authors with these methods. The reaction of Hunter is by o means characteristic for thymine, but is given by carbonyl compounds hich are capable of enolization in Na_2CO_3 solutions (such as acetone).

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[45] D. L. Woodhouse, *Arch. Biochem.* **25,** 347 (1950).

[46] G. Hunter, *Biochem. J.* **30,** 745 (1936).

[47] D. L. Woodhouse, *Biochem. J.* **44,** 185 (1949).

[48] A. Pircio and L. R. Cerecedo, *Arch. Biochem.* **26,** 209 (1950).

3. DETERMINATION OF CYTOSINE AND URACIL

The method of Soodak *et al.*[49] is based on the reduction of arsenotungstic acid (uric acid reagent) by brominated cytosine and uracil. Solutions containing no more than 5 μg. per cc. of cytosine can still give a clearly visible color in mixtures of the two pyrimidines; both of them can be determined after quantitative removal of cytosine by a zeolite, Decalço. Thymine and purines do not interfere.

[49] M. Soodak, A. Pircio, and L. R. Cerecedo, *J. Biol. Chem.* **181,** 713 (1949).

CHAPTER 10

Isolation and Composition of the Deoxypentose Nucleic Acids and of the Corresponding Nucleoproteins

ERWIN CHARGAFF

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I. Introductory Remarks

The early history of the discovery of the deoxypentose nucleic acids (DNA) has been mentioned in Chapter 1 of this book. A much lengthier, and in part pleasantly autobiographical, treatment will be found in Levene's admirable monograph;[1] and the later stages were touched upon, though in considerably less detail, by several authors.[2-4] The fascinating correspondence which forms part of the collected papers of F. Miescher[5] must, however, be specially mentioned in a review of this subject.

An account—not practicable in the present framework—of the evolutionary sequence of our understanding of the nucleic acids would not be uninstructive: it would show how often in the natural sciences, even behind seemingly trivial experiments, there lies an entire vision of nature; it would demonstrate that the answers which the investigator receives often are contained in the questions he asks. As long as, in the field under consideration here, feeble and faltering questions were asked, the answers were vague;

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[1] P. A. Levene and L. W. Bass, "Nucleic Acids." The Chemical Catalog Company, New York, 1931.

[2] J. M. Gulland, G. R. Barker, and D. O. Jordan, *Ann. Rev. Biochem.* **14**, 175 (1945).

[3] E. Chargaff and E. Vischer, *Ann. Rev. Biochem.* **17**, 201 (1948).

[4] E. Chargaff, *Experientia* **6**, 201 (1950).

[5] "Die histochemischen und physiologischen Arbeiten von Friedrich Miescher. Gesammelt und herausgegeben von seinen Freunden," 2 Vols. F. C. W. Vogel, Leipzig, 1897.

but when the direction of the quest became reasonably well defined, the results sharpened in definition. Though we still are very far from a solution of the many problems that keep multiplying as our knowledge progresses, it would be hazardous to erect a sign saying "Ignorabimus." But much is left to future generations.

The deoxypentose nucleic acids have often been isolated by way of the nucleoproteins. That the use of these conjugated proteins also has provided an encouraging procedure for nucleic acid fractionation will be mentioned later. In other cases direct isolation methods for the preparation of the nucleic acids from a variety of tissues have been employed. These different procedures will be described after a brief consideration of the nucleoproteins themselves. These sections will in turn be followed by a discussion of the properties and of the composition, in both its qualitative and quantitative aspects, of the deoxypentose nucleic acids. Their fractionation and methods for their structural investigation will be taken up next; and, finally, a provisional summation will be attempted.

II. Deoxypentose Nucleoproteins

1. General

Beneath the simple textbook definition of a nucleoprotein—a combination between a protein and a nucleic acid—there lies an ocean of uncertainties. Studies on nucleoproteins lead right into one of the most neglected, because most difficult, fields of present-day biochemistry, namely, the conjugated proteins. The deoxynucleoproteins are usually defined as conjugated proteins in which the union between the deoxypentose nucleic acid, functioning as a prosthetic group, and the protein is mediated by electrostatic attraction or by secondary valence forces.[6-8] It is, however, as was pointed out some years ago in a discussion of the cognate problem of the lipoproteins,[9] extremely difficult to distinguish between these two types of combination when dealing with macromolecules. Moreover, a decision will have to be made in every case whether what has been isolated really preexisted in the cell as a conjugated protein or whether it was produced by the combination between solutes fortuitously present in the same cell extract, thus simulating a definite compound. One of the attributes of a conjugated protein is that it must differ in some of its properties from a mere mixture of its components. A genuine nucleoprotein would, therefore, have to be regarded as a geometrically unique compound between two giant polyampho-

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[6] J. P. Greenstein, *Advances in Protein Chem.* **1,** 209 (1944).

[7] E. Chargaff, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 28 (1947).

[8] E. Chargaff, *in* "Some Conjugated Proteins," p. 36. Rutgers University Press, New Brunswick, New Jersey, 1953.

[9] E. Chargaff, *Advances in Protein Chem.* **1,** 1 (1944).

lytes; and a most extensive comparison of the properties of artifacts and of the supposedly genuine nucleoprotein would have to precede a decision. Such comparisons, extremely difficult in the absence of suitable biological tests, have not yet been carried out to any extent, though a beginning has recently been made by Crampton *et al.*[10]

If, as will be discussed later, the deoxypentose nucleic acid fraction of a given nucleus is composed of a large number of differently constituted individuals, a nucleoprotamine or a nucleohistone preparation must be assumed to comprise many different conjugated proteins; a number that will be even greater if not only different nucleic acids, but also different proteins enter into partnership. The reassociation of the various nucleic acid and protein moieties that may take place, if dissociation has occurred in the course of isolation, will introduce additional complications. In this field, simplification may relieve the mind, but will not ease the burden, of the investigator. In any event, an inspection of the literature will lead to the conclusion that very few, if any, entities that can be considered as genuine deoxypentose nucleoproteins have been isolated. As is also true of the corresponding ribonucleoproteins (see Chapter 11) only those structures that are characterized by a high degree of complexity, bordering on organization, and by specific biological activity, the viruses and phages, can safely be so designated.

Whether the major part, or all, of the deoxypentose nucleic acids occurs in the cell in the form of nucleoproteins cannot yet be stated. But there is little doubt that when nucleic acids and proteins are isolated together, i.e., in cell extracts, the deoxypentose nucleic acid can be freed of protein only in the presence of a high salt concentration.[10] Under these conditions a dissociation of the conjugated protein may have taken place;[11] and a nucleoprotein recovered after the exposure of its solution to a high electrolyte concentration may represent an artifact, a protein nucleate.[3] A comparison of several properties of nucleohistone preparations isolated in the absence of electrolytes with those of specimens that had been in contact with salt revealed a number of differences, but they were of a relatively minor nature.[10] The entire field of conjugated proteins suffers from our incomplete knowledge of the conditions governing interactions between polyelectrolytes and of the geometry of the resulting products.

Before discussing, in the following sections, several representative nucleoproteins, especially those that have served for the isolation of deoxypentose nucleic acids, reference should be made to the reviews on nucleoproteins written by Greenstein[6] and Markham and Smith.[12]

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[10] C. F. Crampton, R. Lipshitz, and E. Chargaff, *J. Biol. Chem.* **206**, 499 (1954).

[11] I. Bang, *Beitr. chem. Physiol. u. Path.* **4**, 331 (1904); **5**, 317 (1904).

[12] R. Markham and J. D. Smith, *in* "The Proteins" (H. Neurath and K. Bailey, eds.), Vol. II, p. 1. Academic Press, New York, 1954.

2. Classification

As in the case of the lipoproteins,[9] the classification of conjugated proteins in general must be based on the nature of both the prosthetic group and the protein. The former division is implicit in the use of the terms deoxypentose and pentose nucleoproteins. Beyond this it is not yet possible to go, since in no instance, not even in that of the viruses, can the presence of a single nucleic acid individual, homogeneous with respect to both structure and function, be affirmed.

As regards the protein moiety, a crude classification would distinguish three principal groups: (a) The nucleoprotamines, discovered by Miescher[5,13] in ripe sperm nuclei of the salmon and occurring in ripe spermatozoa of many fish genera. (b) The nucleohistones, observed by Kossel[14] in bird erythrocytes and investigated in greater detail in the form of the prototypal thymus nucleohistone by Lilienfeld,[15] Huiskamp,[16] and Bang,[11] though here again the first observations are due to Miescher.[5] The extent of occurrence of nucleohistones in the nuclei of mammalian cells is not yet clearly defined, nor is it possible to draw an entirely satisfactory demarcation line between these two types of nucleoprotein. The spermatozoa of sea urchins and mollusks appear to contain basic proteins of a more complicated composition than is usually encountered in the protamines;[17] rooster sperm seems to contain a protamine.[18,19] The most comprehensive monograph on the basic proteins still is that of Kossel;[20] a shorter modern treatment has been given by Felix;[21] the amino acid composition has been discussed by Tristram.[22]

The third, and in some respects perhaps the most interesting, group are (c) the nucleoproteins in the proper sense of this term. In this type of compound the deoxypentose nucleic acid is bound, most likely by secondary valence forces, to a protein lacking the basic properties of the protamines and histones. A nucleoprotein of this type has been isolated from avian tubercle bacilli.[23] It is in contrast to the nucleoprotamines and nucleohis-

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[13] F. Miescher, *Hoppe-Seylers Med.-chem. Untersuchungen* **4,** 441 (1871).

[14] A. Kossel, *Z. physiol. Chem.* **8,** 511 (1884).

[15] L. Lilienfeld, *Z. physiol. Chem.* **18,** 473 (1894).

[16] W. Huiskamp, *Z. physiol. Chem.* **32,** 145 (1901).

[17] T. Hultin and R. Herne, *Arkiv Kemi, Mineral. Geol.* **26A,** No. 20 (1948).

[18] M. M. Daly, A. E. Mirsky, and H. Ris, *J. Gen. Physiol.* **34,** 439 (1951).

[19] H. Fischer and L. Kreuzer, *Z. physiol. Chem.* **293,** 176 (1953).

[20] A. Kossel, "The Protamines and Histones." Longmans, Green and Co., London and New York, 1928.

[21] K. Felix, *in* "Physiologische Chemie" (B. Flaschenträger and E. Lehnartz, eds.), Vol. I, p. 709. Springer, Berlin, Göttingen, Heidelberg, 1951.

[22] G. R. Tristram, *in* "The Proteins" (H. Neurath and K. Bailey, eds.), Vol. I, p. 181. Academic Press, New York, 1953.

[23] E. Chargaff and H. F. Saidel, *J. Biol. Chem.* **177,** 417 (1949).

tones soluble in isotonic salt solutions. Whether these nucleoproteins occur only in microorganisms cannot yet be stated. A histone has been claimed to be present in the nucleoprotein fraction of Type III pneumococci.[24] It is not impossible that the protein moiety of these nucleoproteins has a more complicated amino acid composition than have the basic proteins of low molecular weight. Moreover, the existence of similar nucleoproteins in the nuclei of higher organisms cannot be excluded, although complications arising from the contamination of the preparations with cytoplasmic material may account for some of the results. While nucleoprotamine appears to constitute almost the entire mass of defatted fish sperm nuclei,[25-27] many other varieties of nuclei include tryptophan-containing proteins.[24,28,29] The nucleoproteins of some of the animal and bacterial viruses possibly also belong to this class; but, the chemical information available at present is not sufficient for a decision.

It is quite obvious that we are very far from a meaningful classification of the types of deoxypentose nucleoprotein occurring in cell nuclei, especially with regard to changes in the composition of the protein moiety taking place during development and in the several phases of the mitotic cycle.

3. ISOLATION

Two principal methods, both foreshadowed in the pioneering work of Miescher,[5] have been used for the preparation of nucleoprotamines and of nucleohistones, i.e., complexes in which the protein partner carries a positive charge. They are based on (a) extraction with solutions of low ionic strength;[15,16,30] (b) extraction with strong salt solutions.[24,25,31] In recent years, the studies of Hammarsten[30] and of Mirsky and Pollister[31] were of particular importance in providing the impulse for the isolation of a large number of highly polymerized preparations of deoxypentose nucleic acid. As already has been mentioned, certain bacterial nucleoproteins, which appear to belong to an entirely different type of nucleoprotein, can be extracted with dilute buffers and fractionated by conventional means.[23]

[24] A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.* **30,** 117 (1946).

[25] A. W. Pollister and A. E. Mirsky, *J. Gen. Physiol.* **30,** 101 (1946).

[26] K. Felix, H. Fischer, A. Krekels, and R. Mohr, *Z. physiol. Chem.* **287,** 224 (1951).

[27] K. Felix, H. Fischer, A. Krekels, and R. Mohr, *Z. physiol. Chem.* **289,** 10 (1951).

[28] E. Stedman and E. Stedman, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 224 (1947).

[29] A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **31,** 1, 7 (1947).

[30] E. Hammarsten, *Biochem. Z.* **144,** 383 (1924).

[31] A. E. Mirsky and A. W. Pollister, *Proc. Natl. Acad. Sci. U. S.* **28,** 344 (1942).

a. Extraction with Solutions of Low Ionic Strength

This procedure, which has been used repeatedly for the isolation of nucleoproteins,[10 32-36] in particular of calf thymus nucleohistone, has the advantage of avoiding the exposure of the conjugated protein to high salt concentrations, and therefore to dissociating conditions, in the course of its preparation. There exists, however, the danger of a partial enzymic degradation of the nucleic acid brought about by the release of nucleases. Attempts are usually made, based on the behavior of the pancreatic deoxyribonuclease (see Chapter 15), to suppress the enzymic attack by the use of arsenate, citrate, or such chelating agents as sodium ethylenediamine tetraacetate. There is, however, some doubt as to the role of these complex-forming agents, owing to the existence of nucleases that differ in their requirements from the pancreatic enzyme,[37-40] and their efficacy may have to be explained in a different manner.[35]

A preparation, at a low electrolyte concentration, of calf thymus nucleohistone is given as the first example, and the nucleoprotein of tubercle bacilli as the second.

(*1*) *Preparation of Calf Thymus Nucleohistone.*[10] Trimmed calf thymus was obtained fresh at the slaughter-house, chilled immediately, and processed without delay. All subsequent operations were performed at 4–6°. Fifty-gram portions of tissue were triturated for 30 seconds in a high-speed mixer equipped with cutting blades with 50 cc. of an ice-cold mixture of aqueous 0.1 M NaCl and 0.05 M sodium citrate (previously adjusted to pH 7). The supernatant fluid resulting from centrifugation at 2000 × g for 30 minutes was discarded and the suspension of the sediment in 100 cc. of saline–citrate once more centrifuged. The sediment was washed three times by thorough resuspension and centrifugation, each time with 50 cc. of distilled water (previously adjusted to pH 7 by being made about 0.0004 M with respect to $NaHCO_3$), in order to remove electrolytes. During the final washing the sediment swelled, but yielded less than 0.5% of its total phosphorus to the supernatant fluid. The gelatinous sediment then was blended (15 seconds in the high-speed mixer) with 250 cc. of distilled water (pH 7) and shaken overnight. The extremely viscous mixture was again briefly stirred in the high-speed mixer and centrifuged for 30 minutes at 2000 × g. The P contents of the very viscous, opalescent supernatant fluids averaged 430 μg.

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[32] K. G. Stern, G. Goldstein, J. Wagman, and J. Schryver, *Federation Proc.* **6,** 296 (1947).

[33] D. C. Gajdusek, *Biochim. et Biophys. Acta* **5,** 397 (1950).

[34] K. G. Stern, G. Goldstein, and H. G. Albaum, *J. Biol. Chem.* **188,** 273 (1951).

[35] M. H. Bernstein and D. Mazia, *Biochim. et. Biophys. Acta* **10,** 600 (1953).

[36] J. A. V. Butler, P. F. Davison, D. W. F. James, and K. V. Shooter, *Biochim. et Biophys. Acta* **13,** 224 (1954).

[37] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **180,** 727 (1949).

[38] M. E. Maver and A. E. Greco, *J. Biol. Chem.* **181,** 861 (1949).

[39] M. Webb, *Nature* **169,** 417 (1952).

[40] M. Webb, *Exptl. Cell Research* **5,** 27 (1953).

per cc. (about 90% of total P in the mixture). The reextraction of the insoluble residu with water or *M* NaCl yielded negligible amounts of P.

For the precipitation of the nucleohistone the aqueous solution was made 0.15 *M* with respect to NaCl by the addition of 5.66 vol. of 0.177 *M* NaCl. The resulting precipitate was collected 30 minutes later by centrifugation and washed on the centrifuge with 0.15 *M* NaCl and then, very briefly, with a very small amount of distilled water. The weight ratios of protein to P in such preparations were around 12.

The extinction, expressed as $\epsilon(P)$,[41] of nucleohistone thus prepared is around 6600, regardless of the presence or absence of salt and close to the figure found for most specimens of sodium deoxypentose nucleate. [See below, Section IV.1, and also *Beaven, Holiday, and Johnson*, Chapter 14.] This is of interest, since it would have been conceivable that the extinction of a nucleoprotein would be less than that of the free nucleic acid, if the purines and pyrimidines were involved in the architecture of the intact conjugated protein in such a manner as to bring about the suppression of some of the chromophores.[42]

Nucleohistone is soluble at a very low ionic strength. With increasing salt concentration, the solubility first decreases steeply, reaching a minimum in isotonic solutions, and then rises gradually. Though variations occur, freshly prepared nucleohistone is, in general, soluble in NaCl solutions stronger than 0.7 or 0.8 *M*. Small amounts of phosphorylated compounds that can be extracted from nucleohistone by 0.02 to 0.15 *M* NaCl solutions usually lack the typical nucleic acid spectrum and represent impurities.

(*2*) *Preparation of the Nucleoprotein of Avian Tubercle Bacilli.*[23] A mixture of 25 g. of ether-washed dry bacilli and 100 g. of washed, very fine, Pyrex glass powder (diameter 3 μ) was moistened with 0.1 *M* borate buffer (pH 8.3) and divided into eight portions, each of which was ground for 30 minutes in a mortar. The ground cells were united, shaken in a refrigerator with 500 cc. of the borate buffer for 2 days, and centrifuged for 30 minutes at 1900 × *g*. The strongly opalescent, slightly yellow supernatant was decanted through a filter. The centrifugation residue was washed with 500 cc. of borate buffer which then served for the extraction of a second 25-g. portion of disintegrated bacilli. In this manner a total of 100 g. of organisms was processed. The extracts were dialyzed against running water for 48 hours, concentrated by pervaporation to about one-third of the original volume, and again dialyzed against ice-cold distilled water for 72 hours. Ethyl mercurithiosalicylate was added (0.01%) and the bacterial glycogen[43] removed by sedimentation at 31,000 × *g*. The supernatants were once more dialyzed, and the crude nucleoprotein fraction was recovered by evaporation of the frozen solution in a vacuum (yield 2.7 g.). The yields varied for different preparations between 2.4 and 3.4% of the starting material. The crude nucleoprotein fractions, which could easily be dispersed in water or buffer solutions, gave positive reactions for deoxypentose with diphenylamine and cysteine (see Chapter 9) and also e Feulgen reaction (see Chapter 17). They were not precipitated when their solution *M* sodium chloride was diluted to a molarity of 0.15. They were precipitated by tri- loroacetic acid and gave positive biuret, xanthoproteic, Millon, and Hopkins-Cole eactions. Electrophoresis revealed the presence of three components.

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[41] E. Chargaff and S. Zamenhof, *J. Biol. Chem.* **173,** 327 (1948).

[42] B. Magasanik and E. Chargaff, *Biochim. et Biophys. Acta* **7,** 396 (1951).

[43] E. Chargaff and D. H. Moore, *J. Biol. Chem.* **155,** 493 (1944).

For further purification, the dialyzed crude nucleoprotein solution (190 cc.), con- aining a total of 265.1 mg. of N and 16.1 mg. of P, was brought to pH 4.3 by the ddition of 2% acetic acid. The mixture in which a precipitate appeared immediately as chilled for 3 hours and centrifuged. The sediment was washed with ice-cold 0.05 M itrate buffer of pH 4.3, dissolved in borate buffer (pH 8.4), and dialyzed. To this olution an equal volume of saturated ammonium sulfate solution was added and the recipitate removed by centrifugation. The supernatant solution was, after prolonged ialysis, evaporated in the frozen state in a vacuum. The purified nucleoprotein ormed a white fiber felt and weighed 0.29 g. It contained N 12.1%, P 3.2% (N:P per ent ratio 3.8), accounting for about 60% of the phosphorus of the crude nucleopro- ein fraction. The absorption spectra in the ultraviolet of both the crude and the urified nucleoprotein fractions are reproduced in Fig. 1.

. *Extraction with Strong Salt Solution*

This procedure, very widely used as the first step in the isolation of eoxypentose nucleic acids (see below, Section III.2), has also been much

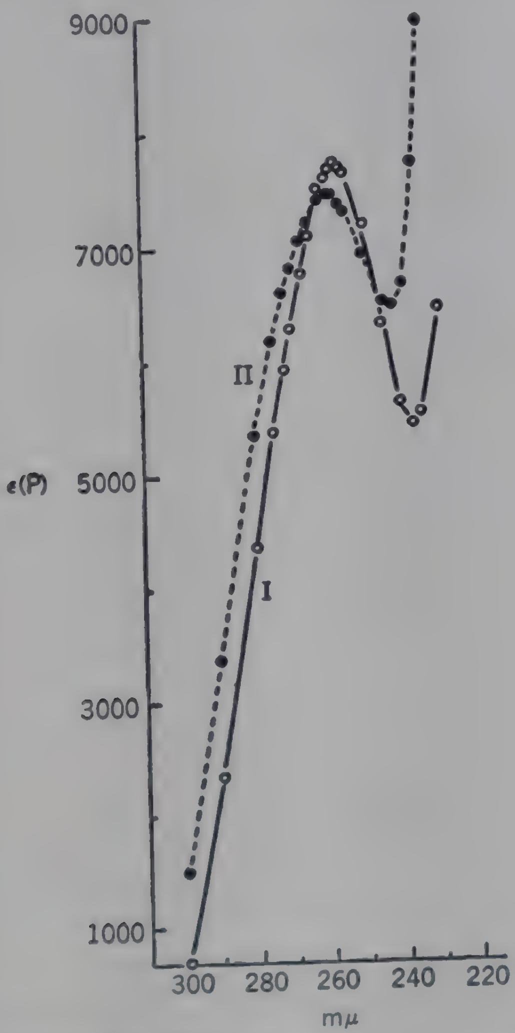

FIG. 1. Ultraviolet absorption spectra of deoxypentose nucleoprotein of avian tubercle bacilli in 0.03 *M* borate buffer at pH 7.9. Curve I, purified nucleoprotein; Curve II, crude nucleoprotein. (Taken from Chargaff and Saidel.[23])

employed for the preparation of nucleoprotamine[24,26,44] and nucleohistone.[25,31,45-50] There exists, however, considerable evidence that, as regard the physical intactness of the nucleoproteins,[51] this method is inferior to tha described in the preceding section (II.3.*a*) since, at any rate in molar o stronger sodium chloride solutions (*M* glycine is considered less harmful[51]) a far-reaching separation between nucleic acid and protein takes place.[10,11] [24,25,30,52,53] This can best be shown under experimental conditions that lea to the removal of one of the partners.[10,25] On the other hand, an advantag of this procedure may be seen in the suppression of the activity of deoxyribonuclease in strong salt solutions.[54,55]

The preparation of a nucleoprotamine is given as an example.

(*1*) *Preparation of the Nucleoprotamine of Trout Sperm.*[25] The operations were carried out in a cold room. Before extraction the freshly collected trout sperm were washed with a solution containing in 1000 cc.: 7.8 g. NaCl, 0.664 g. KCl, and 0.687 g. K_2SO_4.[56] After stirring, the suspension was centrifuged at 5000 r.p.m. for 15 minutes. The washed sperm were extracted with *M* NaCl (final concentration after mixing). On adding salt solution, the sperm mass immediately became sticky and gelatinous, so that it appeared at first as if the cells were merely swelling. It was necessary to add a large volume of solution and to stir vigorously in a Waring mixer before it became apparent that the cells were breaking up as their contents passed into solution. For a mass of sperm with a dry weight of 900 mg. the volume of the extraction mixture should be about 500 cc. Even so, the mixture was quite viscous. After vigorous stirring the mixture was centrifuged at 12,000 r.p.m. for 60 minutes. A perfectly clear, viscous supernatant and a scanty residue were obtained. The material extracted from the sperm was precipitated by pouring the supernatant into 6 volumes of water.[58] The

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[44] I. Watanabe and K. Suzuki, *J. Chem. Soc. Japan, Pure Chem. Sect.* **72,** 578, 580, 604 (1951); *Chem. Abstr.* **46,** 3666 (1952).

[45] H. v. Euler and L. Hahn, *Arkiv. Kemi, Mineral. Geol.* **22A,** No. 17 (1946); **23A,** No. 5 (1946).

[46] M. L. Petermann and C. M. Lamb, *J. Biol. Chem.* **176,** 685 (1948).

[47] G. Frick, *Biochim. et Biophys. Acta* **3,** 103 (1949).

[48] M. Fleming and D. O. Jordan, *Discussions Faraday Soc.* **No. 13,** 217 (1953).

[49] E. J. Ambrose and J. A. V. Butler, *Discussions Faraday Soc.* **No. 13,** 261 (1953).

[50] J. M. Luck, D. W. Kupke, A. Rhein, and M. Hurd, *J. Biol. Chem.* **205,** 235 (1953).

[51] K. G. Stern and S. Davis, *Federation Proc.* **5,** 156 (1946).

[52] S. S. Cohen, *J. Biol. Chem.* **158,** 255 (1945).

[53] M. H. Bernstein and D. Mazia, *Biochim. et Biophys. Acta* **11,** 59 (1953).

[54] M. Laskowski, *Arch. Biochem.* **11,** 41 (1946).

[55] M. Kunitz, *J. Gen. Physiol.* **33,** 363 (1950).

[56] It may be of advantage to use a wash fluid containing sodium citrate. The solution used by Chargaff *et al.*[57] contained the following molarities: sodium chloride 0.123, potassium chloride 0.009, potassium sulfate 0.004, sodium citrate 0.01. (Compare also, Bernstein and Mazia.[35])

[57] E. Chargaff, R. Lipshitz, C. Green, and M. E. Hodes, *J. Biol. Chem.* **192,** 223 (1951).

[58] This step is, in my experience, often accompanied by considerable losses, an observation also made by v. Euler and Hahn.[45] If the nucleoprotein is to serve merely as an intermediate in the preparation of deoxypentose nucleic acid, it is preferable to employ precipitation with alcohol. (See below in Section III.2.)

precipitate was in the form of long fibrous strands which could easily be wound around a rod. The fibrous precipitate redissolved in *M* NaCl. Vigorous stirring shortened the time needed to dissolve the precipitate. Any suspended particles were removed by centrifugation of the viscous solution, and the dissolved material was then reprecipitated by pouring the solution into 6 vol. of water. The fibrous material was soluble in *M*, and insoluble in 0.14 *M*, NaCl. Dissolved in *M* NaCl it kept well at 0° without preservative. Dried preparations contained P 5.93%, 6.14%; N 18.1%, 18.4%.

4. Properties

a. Some Chemical and Physical Characteristics

In the absence of a proper biological test procedure no decision on the native state or the attributes of intactness of a nucleoprotein can be made. It is, perhaps, possible to distinguish less badly degraded nucleoproteins from those suffering from excessive mistreatment; but before a systematization of nucleoproteins has been reached—and we are very far from it—such discussions appear pointless. On the other hand, the failure to isolate a nucleoprotein should not be taken, as is sometimes done,[59] as an indication of its absence. The nucleoproteins of fish sperm and calf thymus remain the only easily accessible deoxynucleoproteins, and most of the work has been done with them. Certain points of difference between nucleoproteins and artificially prepared protein nucleates will be mentioned below (Section II.4.*c*).

The solubility properties of freshly prepared nucleohistone have been mentioned in Section II.3.*a*.(1); those of nucleoprotamines seem to be essentially similar.[25] Little is known about salts or complexes of nucleoproteins with heavy metals. The nucleoprotein of tubercle bacilli is completely precipitated by lanthanum.[23]

The recognition of contaminating pentose nucleoproteins is usually based on the available color reactions (Chapter 9) or on the differences in hydrolysis behavior of the respective nucleic acids (Chapters 5 and 16). There exists, unfortunately, no procedure permitting the separation of deoxypentose and pentose nucleoproteins, once they have been isolated together, as for instance in some of the older preparations from liver.[60] It is necessary to undertake the isolation of the deoxypentose nucleoprotein from material that has been freed of pentose nucleoprotein. This can in many, but not all, cases be done by extensive preliminary washing of the cellular material with 0.14 *M* NaCl or, better, with a mixture of 0.1 *M* NaCl and 0.05 *M* sodium citrate. In certain instances a partial centrifugal separation of the extracted nucleoproteins may be feasible. In a number of studies, contamination was, at least in part, avoided by employing isolated nuclei as the source material.[24,26,27,50,61] The separation of the nucleic acids themselves will be discussed later.

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[59] V. L. Koenig, L. Larkins, and J. D. Perrings, *Arch. Biochem. and Biophys.* **39,** 355 (1952).

[60] J. P. Greenstein and W. V. Jenrette, *J. Natl. Cancer Inst.* **1,** 91 (1940).

The nucleic acid content of different nucleoproteins varies, with sourc and preparation, from about 35 to about 60% of the dry weight. In mos nucleohistone preparations it is around 50%. In the nucleoprotamines o trout and of herring Felix *et al.*[26] found a phosphorus-to-arginine ratio of 1 (Compare, also, Vendrely.[62]) The protein content of nucleohistone may b determined by a modified biuret reaction.[10]

The absorption spectrum in the ultraviolet of a nucleoprotein is, i general, identical with that of its nucleic acid moiety (maximum aroun 260 mμ).[23,25,47,50] (Compare Fig. 1.) No depression of the $\epsilon(P)$ of the nuclei acid component is noticeable, nor does the extent of extinction chang materially under conditions of complete dissociation.[10]

Our information on the physical characteristics of nucleoproteins (compare also Chapter 13) is no less meager than that on their chemical properties. Much of what goes under this name in the literature really refers t the nucleic acids themselves. Studies on the sedimentation behavior in the ultracentrifuge of nucleohistone preparations were undertaken by Stern and collaborators,[32,51] but have not yet been described in detail. Several other investigations by means of the ultracentrifuge have also been published[36,46, 47,63,64] as have numerous studies on electrophoretic[33,36,47,48,65,66] and viscosity properties.[46,47,53,63,64,67] Despite several, often very discordant, estimates no molecular weight can safely be stated. No more than passing reference can be made here to studies of X-ray diffraction[68] and scattering,[69] of orientation phenomena in nucleoprotein films,[49] of dielectric properties,[70] and to observations in the electron microscope[71,71a] and in polarized light.[72]

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[61] L. Ahlström, H. von Euler, and L. Hahn, *Arkiv. Kemi, Mineral. Geol.* **22A,** No. 13 (1946).

[62] R. Vendrely and C. Vendrely, *Nature* **172,** 30 (1953).

[63] R. F. Steiner, *Trans. Faraday Soc.* **48,** 1185 (1952).

[64] K. V. Shooter, P. F. Davison, and J. A. V. Butler, *Biochim. et Biophys. Acta* **13,** 192 (1954).

[65] J. L. Hall, *J. Am. Chem. Soc.* **63,** 794 (1941).

[66] Q. Van Winkle and W. G. France, *J. Phys. & Colloid Chem.* **52,** 207 (1948).

[67] R. O. Carter and J. L. Hall, *J. Am. Chem. Soc.* **62,** 1194 (1940).

[68] M. H. F. Wilkins and J. T. Randall, *Biochim. et Biophys. Acta* **10,** 192 (1953).

[69] D. P. Riley and U. W. Arndt, *Nature* **172,** 294 (1953).

[70] L. G. Allgén, *Acta Physiol. Scand.* **22,** *Suppl.* 76 (1950).

[71] H. Fischer, O. Hug, and W. Lippert, *Chromosoma* **5,** 69 (1952).

[71a] W. J. Frajola, M. H. Greider, and J. G. Rabotin, *Biochim. et Biophys. Acta* **14,** 18 (1954).

[72] J. C. White and P. C. Elmes, *Nature* **169,** 151 (1952).

b. *Cleavage and Degradation*

It already has been mentioned (Section II.3.*b*) that there exists considerable evidence that deoxypentose nucleoproteins, or at any rate the nucleoprotamines and nucleohistones, occur in strong salt solutions in a largely dissociated state which has often been regarded as representing no more than a mixture of protein and nucleic acid. This cleaving effect of a high concentration of salt[11] has been employed in what, historically speaking, is one of the most important procedures for the isolation of intact deoxypentose nucleic acid, namely, that of Hammarsten.[30] It is also demonstrated by the observation that the nucleic acid can be freed entirely of protamine by the dialysis of the nucleoprotamine of trout sperm in the presence of *M* NaCl.[25] The addition of alcohol to a salt solution of nucleohistone has a similar effect.[10,52] The fractional dissociation of nucleohistone by increasing concentrations of salt has been studied in some detail by Crampton *et al.*[10] They discuss the possibility that even at a high ionic strength there may remain some residual unbroken links between the nucleic acid and the histone unless one of the partners is being removed continually.

While agents known to break hydrogen bonds, e.g., urea, guanidine, etc., do not seem to have been much employed for preparatory purposes, the detergents have found useful application. After the discovery of the effect of sodium dodecyl sulfate on tobacco mosaic virus,[73] this substance or similar commercial detergent preparations often have served for the isolation of deoxypentose nucleic acid. (See below, Section III.2.*d*.) Sodium deoxycholate has also been used occasionally.[23,74]

The degradation of deoxypentose nucleoproteins has, in contrast to their cleavage, been little studied, no doubt because of their lability and unfavorable solubility properties. Here again, though title or text may not indicate it, much of the work probably deals with the nucleic acids rather than with the nucleoproteins. Nemchinskaya[75] found depolymerization to be retarded when pancreatic deoxyribonuclease acted on a nucleoprotein.

c. *Artifacts*

It is very easy to mix a nucleic acid with a protein, but very difficult to describe the resulting product. The childish urge to pour all into one pot has abated of late and the term nucleoprotein is no longer applied to every concoction. This has been brought about by a realization of the enormous range of variabilities that may prevail when two very large polyampholyt combine with each other.[76]

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[73] M. Sreenivasaya and N. W. Pirie, *Biochem. J.* **32,** 1707 (1938).
[74] O. T. Avery, C. M. MacLeod, and M. McCarty, *J. Exptl. Med.* **79,** 137 (1944).
[75] V. L. Nemchinskaya, *Biokhimiya* **15,** 478 (1950); *Chem. Abstr.* **45,** 3439 (1951).

When a polyacid, such as a deoxypentose nucleic acid, combines with polybase, such as histone or protamine, many different salts or complex may form owing to the multiple possibilities of cross-linking taking plac these may range from a close-knit fabric, the two partners being warp a woof, to a treelike arrangement, in which protein molecules are attache as branches to the nucleic acid trunk (or vice versa), or even to a stoichi metric sandwich, in which the minimum number of protein molecules th can be accommodated is aligned lengthwise on a nucleic acid molecule. most cases, hybrids between all these forms probably will occur. This is field in which poor reproducibility is almost guaranteed, though, stati tically, there may be little difference between the results. The first eve that occurs when the two components are brought together may conditio all subsequent reactions; and the composition of the solvent, the relati and absolute proportions of the partners, and even more the order an rate of their mixing, will influence the quality of the reaction product Moreover, the direction that electrostatic attraction takes first in a give case will not be without influence on the type of secondary valence bond established subsequently. These considerations are of importance for decision whether a nucleoprotein isolated from a tissue may be considere as intact or whether it has been converted to an artifact in the course its preparation owing to a random reassociation of its separated compo nents. (Compare also Section II.1.)

The effect of a series of electrolyte concentrations on several propertie of calf thymus nucleohistone, prepared as described in Section II.3.*a*.(1) has been studied by Crampton *et al.*[10] (See also Section II.4.*a*.) "Native nucleohistone preparations, which had not been exposed to higher tha 0.14 M salt concentrations, were much less soluble in 0.6 M NaCl than i 0.7 M; those that had previously been in contact with M NaCl showed n such differences. The latter preparations also had a higher viscosity. Othe observations that are relevant to this problem will be mentioned later i connection with the discussion of the fractionation of nucleic acids (Sec tion VIII).

The first observations on the formation of a precipitate when nuclei acid and protamine are mixed are due to Miescher.[5] Since that time numer ous studies of the interaction of deoxypentose nucleic acids and protein have been published, of which only a few can be mentioned here. Much o the early work is marred by the employment of degraded preparations Reactions with protamine[77,78] and histone,[30,79,80] egg albumin[30,79-81] an erum albumin,[81-84] and with many other proteins[80,85-88] were studied. One f the most striking differences between a nucleoprotamine or a nucleoistone on the one hand and artifacts prepared by the mixing of a nucleic cid and a basic protein on the other is the solubility of the first-mentioned omplexes in the absence of electrolytes. It now appears that it is possible o prepare nucleic acid – protamine compounds of similar solubility in vater when very dilute aqueous solutions of the components are brought ogether.[78] The combination of deoxypentose nucleic acid with enzymes has een studied in several instances.[86,87] Of particular interest is a recent nvestigation of Shapot[87] dealing with the formation of a complex between leoxyribonucleic acid and pancreatic deoxyribonuclease which is stable in he absence of Mg^{++} ions.[88a] Attention may also be drawn to studies on he protection against heat coagulation that the presence of nucleic acid onfers on proteins.[81,89]

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[76] A. Katchalsky, *Progr. Biophys. and Biophys. Chem.* **4,** 1 (1954).

[77] P. Alexander, *Nature* **169,** 226 (1952).

III. Isolation of Deoxypentose Nucleic Acids

1. GENERAL

The isolation of a cellular constituent of high molecular weight and omplex structure poses several problems of which the most important is he decision whether the isolated preparation may still be regarded as epresentative of the state in which it occurred in the living cell. Strictly peaking, no compound, once it is isolated from the cell, can be considered s native. When a pillar is hacked out of a building, neither pillar nor uilding is left. If the nucleic acids occur in the cell in combination with proteins, and most likely as the prosthetic groups of conjugated proteins, the danger of secondary changes attending their liberation is particularly great, since whatever forces had anchored them to the protein may now have become free to interact. The series of degradative changes to which a nucleic acid is exposed in the course of its isolation will, however, usually be gradual; and, while it may not yet be possible to define the perfect compound, the badly degraded one will, as a rule, be recognized.[90] As in all such cases, one must be satisfied with avoiding the avoidable.

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[78] P. Alexander, *Biochim. et Biophys Acta.* **10,** 595 (1953).

[79] K. B. Björnesjö and T. Teorell, *Arkiv. Kemi, Mineral. Geol.* **19A,** No. 34 (1945).

[80] O. P. Chepinoga and R. Sh. Grosblat, *Ukraïn. Biokhim. Zhur.* **21,** 121 (1949); *Chem. Abstr.* **48,** 4014 (1954).

[81] J. P. Greenstein and M. L. Hoyer, *J. Biol. Chem.* **182,** 457 (1950).

[82] E. Stenhagen and T. Teorell, *Trans. Faraday Soc.* **35,** 743 (1939).

[83] E. Goldwasser and F. W. Putnam, *J. Phys. & Colloid Chem.* **54,** 79 (1950).

[84] E. P. Geiduschek and P. Doty, *Biochim. et Biophys. Acta* **9,** 609 (1952).

[85] A. N. Belozerskiĭ and G. D. Bazhilina, *Biokhimiya* **9,** 134 (1944); *Chem. Abstr.* **39,** 314 (1945).

[86] P. Ohlmeyer, *Biochim. et Biophys. Acta* **4,** 229 (1950).

[87] V. S. Shapot, *Biokhimiya* **17,** 299 (1952); *Chem. Abstr.* **46,** 10238 (1952).

[88] V. L. Ryzhkov and G. I. Loïdina, *Doklady Akad. Nauk S.S.S.R.* **86,** 181 (1952); *Chem. Abstr.* **47,** 1243 (1953).

[88a] The spectral shift in the extinction maximum from 260 to 254 mμ is, according to unpublished experiments with Mr. H. S. Shapiro, not attributable to the formation of the enzyme–substrate complex, as was thought by Shapot.[87] An identical spectrum can be reconstructed by the summation of the individual spectra of deoxyribonucleic acid and deoxyribonuclease, when measured at the pH at which the complex is examined.

[89] C. E. Carter and J. P. Greenstein, *J. Natl. Cancer Inst.* **6,** 219 (1946).

Several physical criteria (viscosity, ultracentrifugal and electrophoretic behavior, light scattering, ultraviolet absorption, etc.) that are in certain cases useful for a decision on the integrity of a preparation are discussed in Chapters 13 and 14. Unfortunately, from most of these tests we may learn how bad a given specimen is, but only rarely how good it is. This applies even more to the use of biological criteria of testing, the retention or loss of transforming activity[74] (see Chapter 27); this expedient is, in its present form, far from being a generally useful guide.[91]

While the early workers in this field, such as Miescher and Hoppe-Seyler (see Chapter 1), conjectured the macromolecular and complex character of the nucleic acids that they were the first to isolate, many of the precepts and safeguards of biochemical etiquette were forsaken by the succeeding generations. This may have been due in part to the powerful influence that the great successes of the organic chemistry of small molecules had on the development of biochemistry.[4] A passage from Levene's book[1] (p. 251) may be instructive: "At that period of their work these investigators were still under the influence of the traditional belief in the unusual lability of the nucleic acids, and they accordingly avoided the use of heat for the liberation of the nucleic acid from the protein." A revival of this, by no means erroneous, traditional belief took place in the last thirty years.[30,92,93]

Deoxypentose nucleic acids usually are isolated from tissues in the form of their sodium salts. The requirements for a satisfactory preparation may be listed as follows: (a) absence of proteins, polysaccharides, lipids, etc.; (b) absence of pentose nucleic acids; (c) freedom from inorganic salts and other easily dialyzable impurities; (d) a phosphorus content not far from 9.2%; (e) a maximum of absorption in the ultraviolet at pH 7 between 257 and 261 mμ with an $\epsilon(P)$ around 6600 (see Chapter 14); (f) fibrous character, a high and nonthixotropic viscosity,[90] presence of streaming birefringence (see Chapter 13). Several other requirements could be added e.g., monodispersity in the ultracentrifuge, electrophoretic homogeneity,

[90] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **186,** 207 (1950).

[91] S. Zamenhof, H. E. Alexander, and G. Leidy, *J. Exptl. Med.* **98,** 373 (1953).

[92] R. Feulgen, *Z. physiol. Chem.* **237,** 261 (1935); **238,** 105 (1936).

[93] R. Signer, T. Caspersson, and E. Hammarsten, *Nature* **141,** 122 (1938).

and, especially, those growing out of the analytical expectations and impressive structural regularities to be discussed later in this article.

The extent to which these several requirements must be fulfilled in a given case will, of course, depend upon the particular purpose. I shall give only two examples. If a specimen of a deoxypentose nucleic acid is to serve for a chemical and analytical study of its composition and of the quantitative distribution of its constituents, a thorough dialysis and lyophilization, both at 0°, will facilitate the sampling. Heating at high temperature should be avoided,[94] except for the determination of moisture. On the other hand, preparations that are to be used for physical and structure studies, and even more those to be tested for biological activity,[91] should not be subjected to dialysis in the absence of electrolytes or to lyophilization. More drastic conditions, such as heat, acid, alkali, or even the excessive use of high-speed mincers,[94a] must be shunned.

2. Preparative Procedures

The preparative procedures for the isolation of highly polymerized sodium deoxypentose nucleate, of which several representative examples will be given, are based on the nucleohistone studies, discussed in detail in Section II, of Bang,[11] Hammarsten,[30] and Mirsky and Pollister.[31] These methods are, in general, applicable to a wide variety of tissues, though special precautions or modifications may occasionally become necessary. Once the nucleoprotein or a mixture of nucleic acid and protein has been extracted, the removal of protein is carried out by methods described by Hammarsten,[30] Sevag,[95,96] and Pirie.[73,97]

a. Extraction with Strong Salt Solution, Deproteinization with Chloroform

(*1*) *Sodium Deoxyribonucleate of Calf Thymus.*[98] Fresh frozen calf thymus glands (54.5 kg.) were minced and suspended in 0.9% sodium chloride (54 l.) and milled to produce a fine suspension. This suspension was centrifuged (6300 r.p.m.) and the solid material resuspended in 0.9% sodium chloride (45.5 l.) and milled and centrifuged as before. The tissues, which were now free of material containing pentose, were suspended in 10% sodium chloride (214 l.) with vigorous mechanical stirring at 0°. At this stage the viscosity of the solution increased considerably. After extraction at 0° for 48 hours, the insoluble material was removed by centrifuging (6300 r.p.m.) and the deoxypentose nucleoprotein precipitated from the resultant solution (pH 6.5) by the addition of an equal volume of industrial methanol. The precipitated solid was washed with 70%, then 100% industrial methanol and dried in a vacuum at room temperature. Yield, 1.69 kg. of a very slightly yellow fibrous solid.

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[94] A. R. Peacocke, *Biochim. et Biophys. Acta* **14,** 157 (1954).

[94a] There exists no really good name for these useful, though sometimes almost too effective, machines ("Waring Blendor," "Turmix," etc.). "Homogenizer"—a horrible *lucus a non lucendo*—must be avoided; "disintegrator" or "macerator" sound too much like science fiction; "blender" is culinary and not sufficiently descriptive. "High-speed mincer" or "high-speed mixer" is perhaps least objectionable.

[95] M. G. Sevag, *Biochem. Z.* **273,** 419 (1934).

[96] M. G. Sevag, D. B. Lackman, and J. Smolens, *J. Biol. Chem.* **124,** 425 (1938).

[97] F. C. Bawden and N. W. Pirie, *Biochem. J.* **34,** 1278 (1940).

[98] J. M. Gulland, D. O. Jordan, and C. J. Threlfall, *J. Chem. Soc.* **1947,** 1129.

The nucleoprotein (500 g.) was powdered to assist solution and dissolved in 10% sodium chloride (45.5 l.) with vigorous mechanical stirring. The solution, which was viscous, was clarified by centrifuging (6300 r.p.m.). To the clear solution was added an equal volume of a mixture of chloroform (35 parts) and amyl alcohol (10 parts), and the mixture was emulsified by rapid mechanical stirring. The emulsion was then separated by centrifuging (DeLaval, model "500", disc-type bowl, 5500 r.p.m.) into three parts: (a) the chloroform – amyl alcohol mixture; (b) a solution containing the sodium salt of deoxypentose nucleic acid and nucleoprotein; (c) a gel of protein hydrochloride and the chloroform – amyl alcohol mixture. The protein gel remained in the bowl of the centrifuge whereas the chloroform – amyl alcohol mixture and the solution of nucleic acid and nucleoprotein were discharged from separate outlets. The last-mentioned solution was again emulsified with the chloroform – amyl alcohol mixture and the process repeated until no gel was formed on emulsification; this required nine emulsifications. The sodium salt of the deoxypentose nucleic acid was precipitated by the addition of an equal volume of methanol, washed free from chloride with 70% methanol, then 100% ethanol, and finally ether, and dried in a vacuum at room temperature. Yields from two 500-g. quantities of nucleoprotein were 130 g. and 150 g. of a white fibrous solid giving negative biuret and Sakaguchi tests.

(*a*) *Small-scale preparation.* A typical preparation from our laboratory is briefly described here. All operations were carried out at about 4°. Two hundred and twenty grams of freshly obtained, well-trimmed calf thymus were cut into small pieces and triturated for 3 minutes in a "Waring Blendor" in 400 cc. of a mixture of 0.1 M NaCl and 0.05 M sodium citrate (adjusted to pH 7). The sediment resulting from centrifugation at 1900 × g for 45 minutes was washed twice more with 350-cc. portions of the same fluid and stirred in a high-speed mixer with 1250 cc. of 10% NaCl. The mixture was kept overnight and centrifuged at 20,000 × g for 45 minutes. The sediment was reextracted for several hours with 800 cc. of 10% NaCl and the mixture centrifuged as above. The combined extracts (about 1600 cc.) were injected in a slow stream into 2 vol. of 95% ethanol. The white strands of nucleohistone were lifted by spooling, drained, and washed with 70% and 80% ethanol. The material was dissolved with the aid of rapid stirring in 2500 cc. of 10% NaCl and the solution freed of protein by being stirred eight times, each time for 150 seconds and being followed by centrifugation at 1900 × g, with one-third volume of chloroform – amyl alcohol (3:1). The supernatant aqueous solution was finally injected into 2 vol. of 95% ethanol and the fibers were spooled, lifted, drained, and washed with 70%, 80%, and 100% ethanol. They were then dried in air or recovered by the lyophilization of their dialyzed aqueous solution. The yield of sodium nucleate amounted to 1.4 to 1.9% of the fresh tissue.

(2) *Sodium Deoxyribonucleate of Yeast.*[41] The isolation of this substance may be regarded as an example of an extreme case, as yeast cells contain almost 50 times as much pentose as deoxypentose nucleic acid.

One kilo of fresh bakers' yeast was washed with 1 l. of 0.1 M sodium citrate (pH 7.3). The yeast cells, recovered by centrifugation, were suspended in 180 cc. of the sodium citrate solution and the thick suspension was passed through an ice-cooled wet crushing mill for bacteria. Each 50-cc. portion was ground for 30 minutes. Folowing dilution with 590 cc. of sodium citrate, the crushed suspension was centrifuged or 2 hours at 4000 r.p.m. Two solid layers sedimented underneath a very opalescent supernatant (915 cc.), a bottom layer of intact cells and an upper layer of cellular ragments. A separate determination showed that about 55% of the cells had actually een crushed.

The upper solid layer consisting of cellular fragments (485 cc.) was suspended in 850 cc. of ice-cold M sodium chloride solution of pH 6.3. The slimy mixture was kept in the refrigerator for 72 hours and then centrifuged at 4000 r.p.m. for 2 hours. The rapid addition of 2 vol. of chilled absolute ethanol to the very viscous supernatant resulted in the precipitation of white threads that could easily be wound on a glass rod and thereby separated from a granular precipitate suspended in the mother liquor. The threads were washed thoroughly by successive immersion in three portions of 73% ethanol, drained, and redissolved in 300 cc. of M sodium chloride with the use of a high-speed mixer.

The turbid solution was freed of protein by being stirred in a high-speed mixer with one-third of its volume of a 9:1 mixture of chloroform – octyl alcohol (for 5 minutes), followed by centrifugation at 4000 r.p.m. for 1 hour. After eight treatments the solution was free of protein and gave no biuret test. At this stage, it was found to contain 0.6 mg. of deoxypentose nucleic acid per cubic centimeter, corresponding to a total of 180 mg. in the original 300 cc. of solution.

The addition of 2 vol. of ethanol to the clear protein-free solution again produced white threads that were spooled on a rod, as described before. An additional amount of fibrous nucleic acid could be recovered by reworking the granular precipitate remaining in the mother liquor.

The threads obtained by the above procedure were found to contain only 19% of deoxypentose nucleic acid; the remainder consisted of ribonucleic acid (64%) and of a polysaccharide. The well-drained threads were taken up in 20 cc. of neutral 10% aqueous calcium chloride and the viscous milky solution was clarified by centrifugation at 20,000 r.p.m. for 2 hours. The sediment was washed twice, under the same centrifugal conditions, with 6-cc. portions of 10% $CaCl_2$. The slow addition of 0.2 to 0.3 vol. of cold absolute ethanol to the combined clear supernatants (32 cc.) brought about the separation of white fibers that were lifted in the usual manner and washed twice with 10% calcium chloride solution containing 0.3 vol. of ethanol.

This fraction was contaminated with about 20% of ribonucleic acid which could be removed by enzymic digestion. To a solution of the precipitate in 45 cc. of 0.2 M sodium borate buffer of pH 7.8, 1.5 mg. of crystalline ribonuclease was added. The solution was subjected to dialysis at room temperature against two changes of 2-l. portions of the borate buffer for 14 hours, against running tap water for 17 hours, and, finally, against several changes of ice-cold distilled water for 26 hours. Then it was again deproteinized, as described before, and evaporated in the frozen state in a vacuum or precipitated with ethanol in the presence of sodium acetate. The sodium salt of deoxypentose nucleic acid thus obtained weighed 105.5 mg. It formed a white fluff which was readily soluble in water, giving a clear viscous solution. The biuret reaction was negative.

b. *Extraction with Strong Salt Solution, Deproteinization by Saturation with Sodium Chloride*

(1) Sodium Deoxyribonucleate of Calf Thymus.[99,100] All operations were carried out at 0° with solutions that were 0.01 M with respect to sodium citrate. 450 g. of

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[99] R. Signer and H. Schwander, *Helv. Chim. Acta* **32**, 853 (1949).

[100] H. Schwander and R. Signer, *Helv. Chim. Acta* **33**, 1521 (1950).

freshly removed thymus were cut into small pieces, mixed with powdered solid CO_2, passed through a meat grinder, and treated in portions with a total of 4 l. of *M* NaCl in a high-speed mixer for a short time. The mixture then was stirred slowly for several days, until the gel was converted to a very viscous, reddish, turbid solution. The dilution with water to a 6-fold volume (830-cc. portions were poured into 4.2-l. portions of water) produced precipitation of the nucleoprotein which was stored in 0.01 *M* citrate. The combined precipitate was washed by decantation with 8 l. of a 1% NaCl solution and twice more dissolved in *M* NaCl and reprecipitated, as described above. It was then dissolved in 5 l. of 10% NaCl and the solution, which was being stirred, adjusted with saturated NaCl solution to a volume of 6 l. and saturated by the addition of solid NaCl. Stirring was continued for 4 days and the mixture then kept for 14 days. A suspension of 480 g. of Celite 545 in 1.5 l. saturated NaCl was added and the mixture stirred vigorously for 24 hours. Following filtration by suction through two filter papers and a layer of Celite (and washing of the filter cake) the filtrate was clarified after the admixture of "Hyflo Super-Cel" by filtration as above. It then was poured into 1.5 vol. of alcohol and the fibers were washed with 70% alcohol, squeezed, and dissolved in 4.5 l. of water while being stirred for several days. Precipitation was repeated, this time with 2 vol. of alcohol, and the fibers were washed with 80%, 96%, and 100% alcohol, and ether, and dried *in vacuo* over H_2SO_4. The sodium nucleate weighed 8 g. (1.8% of the tissue). It was stored in a desiccator over saturated NaCl solution.

c. *Extraction with Water*

A rapid and simple preparation of deoxypentose nucleic acid, which has the advantage of permitting the isolation of histone at the same time, is based on the work of Crampton *et al.*[10] It may start from purified nucleohistone, as described in Section II.3.*a*.(1), or be carried out directly. I describe here a typical isolation often performed in our laboratory (based on experiments of Drs. C. F. Crampton and M. E. Hodes and Miss R. Lipshitz).

(*1*) *Sodium Deoxyribonucleate of Calf Thymus*. All operations were carried out at 4°. Two hundred grams of fresh, well-trimmed calf thymus were triturated for 30 to 45 seconds in 200 cc. of a mixture of 0.14 *M* NaCl and 0.01 *M* sodium citrate in a "Waring Blendor." The mixture was centrifuged at $2000 \times g$ for 20 minutes and the sediment washed three more times with the same wash fluid. The supernatant liquids were discarded. The sediment was distributed with rapid stirring in 850 cc. of distilled water (pH 7) and the mixture kept for 15 to 20 hours. It was stirred briefly to reduce its viscosity and passed quickly through a cooled Sharples supercentrifuge at $33{,}000 \times g$. The effluent was diluted to contain about 2 to 4 mg. of nucleic acid per cubic centimeter and brought to a 2.6 *M* concentration by the addition of solid NaCl. Immediately after the addition of 2 vol. of 95% ethanol the fibers were collected by spooling, pressed free of mother liquor, washed with 66%, 80%, and 95% ethanol and with acetone, and dried in air. The yield corresponded to 80% or better of the phosphorus present in the original aqueous extract. The sodium nucleate usually contained 5% or slightly more of protein. The protein impurity could be removed completely by one treatment with sodium dodecyl sulfate (see the next section).

For a preparation of histone and of nucleic acid free of protein on a large scale 900 g. of fresh calf thymus were minced, washed with citrate – physiol. saline, extracted with water, and the nucleohistone was precipitated at 0.15 *M* NaCl. It was collected in the Sharples centrifuge, dissolved in water, brought to a 2.6 *M* NaCl concentration, and the sodium nucleate precipitated with alcohol. The mother liquor served for the isolation of histone. The sodium nucleate was dissolved in water, treated once with sodium dodecyl sulfate (see below) and recovered as described in the next section. The sodium deoxyribonucleate, dried in air, weighed 23.1 g. (2.6% of the tissue) and contained only 0.65% of protein.

d. Extraction with the Aid of Anionic Detergents

In the recent past, deoxypentose nucleic acid preparations have often been made with the aid of sodium dodecyl sulfate[101] or of detergents of the Duponol type.[102] The use of sodium xylene sulfonate has also been proposed.[103] As in all preparative methods, the type of source material will not be without influence on the success of the isolation. In our own experience, a nucleic acid preparation from sea urchin sperm could not be freed entirely of protein by treatment with a detergent.[104]

(*1*) *Sodium Deoxyribonucleate of Calf Thymus.*[102] Fifty grams of frozen calf thymus were minced in a "Waring Blendor" for 3 minutes[105] in 200 cc. of ice-cold 0.9% NaCl solution containing 0.01 *M* sodium citrate. The sediment resulting from centrifugation at 0° and 2500 r.p.m. for 30 minutes was three more times suspended in citrate–saline, centrifuged as described before, and treated in the "Waring Blendor" for 3 minutes with 1 l. of ice-cold physiol. saline.[106] The mixture was transferred to a large beaker and 90 cc. of the detergent solution (5 g. of sodium dodecyl sulfate or purified Duponol made up to 100 cc. with 45% ethanol) were added. The resulting gel was stirred vigorously at room temperature for 3 hours during which time it turned gradually into a very viscous solution. The NaCl concentration then was brought to 1 *M* by the addition of 55 g. of salt,[107] stirring was continued for 10 minutes, and the mixture was centrifuged at 0° and 2500 r.p.m. (or at a higher speed) for 3 hours.[108] The crude sodium nucleate was precipitated by the addition to the supernatant fluid of an equal volume of 95% ethanol, the fibrous precipitate was lifted by spooling, pressed out, and washed 3 times with 95% ethanol and then with acetone until the washings were no longer cloudy. It was dried in air (yield about 2 g.).[109] The crude product was suspended in 700 cc. of distilled water and brought into solution by being stirred rapidly at room temperature; 63 cc. of the detergent solution was added and the mixture was stirred for 1 hour. The mixture was made 1 *M* by the addition of 45 g. of NaCl, centrifuged at 13,000 r.p.m. for 1 hour, and the nucleate precipitated with ethanol from the supernatant and washed as before. It was dissolved once more in water and the solution adjusted to 0.14 *M* NaCl and centrifuged at 13,000 r.p.m. for 1 hour. The NaCl concentration of the supernatant fluid was increased to 1 *M* and the sodium deoxyribonucleate precipitated by the slow addition, with stirring, of an equal volume of 95% ethanol. The fibers, washed with ethanol and acetone and dried in air, weighed about 1.3 to 1.4 g.

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[101] A. M. Marko and G. C. Butler, *J. Biol. Chem.* **190,** 165 (1951).

[102] E. R. M. Kay, N. S. Simmons, and A. L. Dounce, *J. Am. Chem. Soc.* **74,** 1724 (1952).

[103] N. S. Simmons, S. Chavos, and H. K. Orbach, *Federation Proc.* **11,** 390 (1952).

[104] E. Chargaff, R. Lipshitz, and C. Green, *J. Biol. Chem.* **195,** 155 (1952).

[105] A grinding period of 30 to 45 seconds is sufficient in our experience. Excessively long treatment in the "Waring Blendor" should, in general, be avoided.

[106] For the preparation of large quantities we have found it advantageous to extract the nucleohistone at this stage with 10% NaCl solution and to precipitate with alcohol. The nucleohistone can be stored in the cold under aqueous alcohol and then be processed as described in the following.

[107] Care must be taken not to exceed this NaCl concentration, in order to prevent the precipitation of the detergent.

[108] The mixture must not be stored in the cold at this stage, since it may jellify. We centrifuge in the cold for 30 minutes at 13,000 r.p.m. in a rotor that has not been precooled or, if large quantities are to be processed, in a Sharples supercentrifuge at 37,000 r.p.m. with a jet delivering 1 l in 15 minutes. Filtration of the mixture[101] is not recommended; it is accompanied by losses and requires a disproportionate amount of patience.

e. *Comparison of Different Isolation Procedures*

A systematic investigation of the merits of the several procedures still remains to be carried out, except for some preliminary information on relative yields which has recently been provided by Frick[110] and a few findings published by Schwander and Signer.[100] Frick compared the methods of Gulland *et al.*[98] (see Section III.2.*a.*(1)), of Hammarsten[30] (compare the modification by Schwander and Signer[100] described in Section III.2.*b.*(1)), and of Kay *et al.*[102] (see Section III.2.*d.*(1)). His conclusion, only partially borne out by the experience of this laboratory, was that the Gulland procedure gave yields amounting to only 5 to 10% of the quantity of nucleic acid present in the nucleohistone serving as the starting material; that the Hammarsten procedure led to a product in good yield, but of a relatively high protein content; and that the method of Kay *et al.* afforded the greatest yield, but produced some degradation, as judged from ultraviolet absorption.

It is quite clear that yield is only one of several criteria. The constancy of physical and chemical characteristics of preparations isolated by different procedures has been investigated only to a very limited extent; but the rather extensive experience of our laboratory has failed to reveal divergences in composition or extinction that were due to the preparative methods, if the precautions outlined in the preceding sections were observed. (Compare, for instance, the study on deoxypentose nucleic acids of mammalian origin.[111]) As regards biological properties it is worth mentioning that treatment with neither sodium deoxycholate and chloroform[74] nor an anionic detergent[91] appeared to destroy the transforming activity.

The content in deoxyribonucleic acid of fresh calf thymus may be estimated as being in the neighborhood of 2.5%. [See *Leslie*, Chapter 16.] Gulland *et al.*[98] report an average yield of 0.87% for a preparation made on an almost industrial scale. When the sodium nucleate is prepared by his procedure in less gigantic dimensions and with efficient washing of the chloroform gels to overcome entrapment, the yields range, in our experience, from 1.4 to 1.9%. Schwander and Signer[100] record 1.8%, Kay *et al.*,[102] 2.6 to 2.8%. The latter method and the procedure described above under Section III.2.*c*.[10] (2.6% yield) appear to permit the greatest recovery; but all the processes outlined in detail before are, on the whole, quite satisfactory, at any rate for many mammalian organs and for fish sperm. Some special cases will be mentioned below. Although the method outlined by Schwander and Signer[100] consumes much more time than the others, it should not be forgotten that one of their nucleate preparations has afforded the best X-ray photographs.[112,113] [See *Jordan*, Chapter 13.]

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[109] If the freshly precipitated and washed fibers are cut into very small pieces before being suspended in distilled water, rapid stirring for 1 to 2 hours will be sufficient to bring them into solution.

[110] G. Frick, *Biochim. et Biophys. Acta* **13,** 41, 374 (1954).

[111] E. Chargaff and R. Lipshitz, *J. Am. Chem. Soc.* **75,** 3658 (1953).

f. Miscellaneous Procedures and Applications

The isolation methods discussed above, which were developed principally for calf thymus, may be applied not only to thymus tissue of other genera (sheep, pig, man)[111] but also to several other mammalian organs (e.g., spleen,[114] kidney,[115] thyroid[111]) without essential changes. For the preparation of satisfactory specimens of deoxypentose nucleic acid from other organs, in particular liver,[111,116] exhaustive preliminary washing of the minced tissue, to remove pentose-containing material, will be necessary; and such specimens may have to be subjected to special purification procedures after their isolation, in order to free them entirely of pentose nucleic acid, as will be discussed below. Nucleated erythrocytes have also served as a source,[115] as has hen's egg white.[117,118] Occasional observations on the isolation of nucleic acids from malignant tissue are likewise recorded in the literature.[111,116,119-122]

While fish spermatozoa[57,115,123] and testes,[115,119,124] and also sea urchin

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[112] M. H. F. Wilkins, A. R. Stokes, and H. R. Wilson, *Nature* **171,** 738 (1953).

[113] R. E. Franklin and R. G. Gosling, *Nature* **171,** 740 (1953).

[114] E. Chargaff, E. Vischer, R. Doniger, C. Green, and F. Misani, *J. Biol. Chem.* **177,** 405 (1949).

[115] M. M. Daly, V. G. Allfrey, and A. E. Mirsky, *J. Gen. Physiol.* **33,** 497 (1950).

[116] E. Chargaff, B. Magasanik, E. Vischer, C. Green, R. Doniger, and D. Elson, *J. Biol. Chem.* **186,** 51 (1950).

[117] H. L. Fraenkel-Conrat, W. H. Ward, N. S. Snell, and E. D. Ducay, *J. Am. Chem. Soc.* **72,** 3826 (1950).

[118] H. Fraenkel-Conrat and E. D. Ducay, *Biochem. J.* **49,** xxxix (1951).

[119] S. G. Laland, W. G. Overend, and M. Webb, *J. Chem. Soc.* **1952,** 3224.

[120] P. C. Elmes, J. D. Smith, and J. C. White, *IIe Congrès International de Biochimie, Résumés des communications*, Paris **1952,** 7.

[121] I. Asimov and R. R. Simon, *Federation Proc.* **12,** 172 (1953).

[122] D. L. Woodhouse, *Biochem. J.* **56,** 349 (1954).

[123] G. R. Wyatt, *Biochem. J.* **48,** 584 (1951).

[124] C. F. Emanuel and I. L. Chaikoff, *J. Biol. Chem.* **203,** 167 (1953).

spermatozoa,[104,115,123] offer no particular difficulties of isolation, except for the occasional need of 2 or 3 M NaCl solutions for the extraction of the nucleoprotein,[104,115] the preparation of the intact deoxypentose nucleic acids of mammalian sperm often is no easy task. In what appears to be the first isolation of deoxypentose nucleic acid from human sperm[125] it was necessary to treat the washed and defatted spermatozoa with crystalline trypsin (free of deoxyribonuclease) before the extraction of nucleic acid could be performed. In this manner, specimens of a high degree of polymerization were obtained. In later experiments with ram and bull sperm a rather unsatisfactory expedient, viz., extraction with KOH, seems to have been employed.[123,126] It is known that even very strong salt solutions fail to extract nucleoproteins from mammalian spermatozoa;[127] but whether this failure is due to unusual properties of the nucleoproteins themselves or to obstruction by another protein that is removed by tryptic digestion[125] cannot be decided.

Our information on deoxypentose nucleic acids from plant tissues is regrettably meager. It is almost entirely limited to a few preparations from several varieties of plant germ which since the pioneering experiments of Kiesel and Belozersky[128] and the later studies of Feulgen *et al.*[129] have been known as good sources. Belozerskiĭ and his colleagues[130-132] have been particularly interested in the isolation of nucleoproteins, mostly mixtures of the deoxypentose and pentose varieties, from plant sources. The contamination with pentose nucleic acid of the deoxypentose nucleic acid specimens isolated in the recent past from wheat or rye germ[115,119,123,133,134] is an obstacle to structural and analytical studies which appears to have been overcome only rarely, either by degradation with alkali[123] or by special purification[134] (see Section III.2.*h.* below).

g. Nucleic Acids of Microorganisms and Viruses

Unicellular organisms present a special problem as regards the isolation of deoxypentose nucleic acids. No systematic treatment of preparative ethods can be offered though some progress has doubtless been made uring the time elapsed between the first and the second of two previous views on this subject.[7,135] The uncertainties are in part due to the diffi- lty of preparing sufficient starting material and of disintegrating it in a itable manner; but they are to an even larger extent inherent in our ck of knowledge of microbial nucleoproteins and in the scarcity of informa- on on the topical separation of the various elements composing the internal ructure of the microbial cell. Furthermore, the frequent presence of eoxypentose nucleases of different and largely unknown properties[37] often nders the preparation of intact specimens very difficult.

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[125] S. Zamenhof, L. B. Shettles, and E. Chargaff, *Nature* **165,** 756 (1950).

[126] T. Mann, *in* The biochemistry of fertilization and the gametes, *Biochem. Soc. Symposia (Cambridge, Engl.)* **No. 7,** 11 (1951).

[127] L. E. Thomas and D. T. Mayer, *Science* **110,** 393 (1949).

[128] A. Kiesel and A. N. Belozersky, *Z. physiol. Chem.* **229,** 160 (1934).

[129] R. Feulgen, M. Behrens, and S. Mahdihassan, *Z. physiol. Chem.* **246,** 203 (1937).

[130] A. N. Belozerskiĭ and I. I. Dubrovskaya, *Biokhimiya* **1,** 665 (1936); *Chem. Abstr.* **31,** 3100 (1937).

[131] A. N. Belozerskiĭ and L. A. Chernomordikova, *Biokhimiya* **5,** 133 (1940); *Chem. Abstr.* **35,** 1457 (1941).

[132] A. N. Belozerskiĭ and M. S. Uspenskaya, *Biokhimiya* **7,** 155 (1942); *Chem. Abstr.* **38,** 131 (1944).

[133] S. Laland, W. G. Overend, and M. Webb, *Acta. Chem. Scand.* **4,** 885 (1950).

[134] G. Brawerman and E. Chargaff, *J. Am. Chem. Soc.* **73,** 4052 (1951).

Two examples have been quoted in detail above, namely, the nucleo- rotein of avian tubercle bacilli[23] (Section II.3.*a*.(2)) and the deoxyribo- ucleic acid of yeast[41,136] (Section III.2.*a*.(2)). From the former, crude eoxypentose nucleic acid could be prepared by treatment with either aturated NaCl solution or sodium deoxycholate.[23] The further purification discussed in the next section.

The first microbial nucleic acids in the course of whose isolation particular ttention was paid to purity and intactness were those from pneumococci ndowed with transforming activity.[74,137,138] [Compare *Hotchkiss*, Chapter 7.] They were isolated by extraction of the cells after lysis with sodium eoxycholate in the presence of sodium citrate and purified by deproteiniza- ion with chloroform (see Section III.2.*a*) and precipitation as the calcium alt.[138] A similar procedure[139] or treatment with anionic detergent[91] (Section II.2.*d*) has served for the isolation of transforming nucleic acid specimens rom *Hemophilus influenzae*. While in these instances the disintegration of he cell was achieved by mild lytic procedures, most microorganisms require n efficient grinding for extraction to take place. Comminution with very ne glass powder or in a wet crushing mill for bacteria was employed in he examples quoted before,[23,41] and a similar procedure, shaking with glass eads (ballotini), led to the extraction of nucleic acid from *Hemophilus ertussis*.[140] From *Escherichia coli*, ground with glass powder, deoxypentose ucleic acid could be extracted with salt solution;[141] for the extraction of *Serratia marcescens* and other organisms 3.5 M aqueous NaCl was em-

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[35] E. Chargaff, *in* Symposium sur le métabolisme microbien, *IIe Congrès International de Biochimie, Paris* **1952,** 41.

[36] E. Chargaff and S. Zamenhof, *J. Am. Chem. Soc.* **69,** 975 (1947).

[37] M. McCarty and O. T. Avery, *J. Exptl. Med.* **83,** 89 (1946).

[38] M. McCarty and O. T. Avery, *J. Exptl. Med.* **83,** 97 (1946).

[39] S. Zamenhof, G. Leidy, H. E. Alexander, P. L. FitzGerald, and E. Chargaff, *Arch. Biochem. and Biophys.* **40,** 50 (1952).

[40] W. G. Overend, M. Stacey, M. Webb, and J. Ungar, *J. Gen. Microbiol.* **5,** 268 (1951).

[41] B. Gandelman, S. Zamenhof, and E. Chargaff, *Biochim. et Biophys. Acta* **9,** 399 (1952).

ployed, in order to suppress nuclease activity.[142,143] Nucleic acids fro acid-fast bacteria have been isolated by extraction with solutions of lo electrolyte concentration,[23,144] with salt and urea solutions,[145] and wi alkali.[146] The latter procedure, which has also been applied to *E. coli*, degrades, of course, the nucleic acids, though it is effective in removi pentose nucleic acid. The deoxypentose nucleic acid of *Sarcina lutea* h also been described,[145,147] as have the nucleic acids from two rickettsia *R. prowazeki* and *R. burneti*.[148]

Very little is known about the preparation of deoxypentose nucleic aci from viruses and even less about the physical state in which the often ve robust isolation procedures have left them. Following observations on t behavior of plant viruses,[149] extraction of bacteriophages with urea sol tions has been used in several instances.[150,151] A milder method, in whic the bacteriophage is broken by being passed through a colloid mill, th producing what has unfortunately been called a "phage grindate," has bee outlined recently.[152] In other cases, the polyhedral and capsule viruses insects, the composition of the deoxypentose nucleic acids was dete mined by the hydrolysis with $HClO_4$ [153] of the total virus preparations.[15

h. Removal of Pentose Nucleic Acid and of Other Impurities

Pentose nucleic acid is in many respects the most troublesome contam nant of deoxypentose nucleic acid preparations. Its presence vitiates spe troscopic measurements and makes impossible the analytical characteriza tion of the specimens. I am listing here some of the procedures that hav been employed for the removal of pentose nucleic acid. I. Separation b electrophoresis: this mild, but laborious and wasteful, method has bee applied in several instances.[23,41,139] II. Fractionation by way of the calciu salts:[41,138] the separation will, however, be far from complete. III. Purifica tion by treatment with crystalline ribonuclease:[41,138] this does not ensur the complete removal of the pentose nucleic acid, which may leave behin

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[142] E. Chargaff, S. Zamenhof, G. Brawerman, and L. Kerin, *J. Am. Chem. Soc.* 72 3825 (1950).

[143] S. Zamenhof, G. Brawerman, and E. Chargaff, *Biochim. et Biophys. Acta* **9,** 40 (1952).

[144] O. Snellman and G. Widström, *Arkiv. Kemi, Mineral. Geol.* **19A,** No. 31 (1945).

[145] A. S. Jones, *Biochim. et Biophys. Acta* **10,** 607 (1953).

[146] J. D. Smith and G. R. Wyatt, *Biochem. J.* **49,** 144 (1951).

[147] S. K. Dutta, A. S. Jones, and M. Stacey, *Biochim. et Biophys. Acta* **10,** 613 (1953)

[148] G. R. Wyatt and S. S. Cohen, *Nature* **170,** 846 (1952).

[149] F. C. Bawden and N. W. Pirie, *Biochem. J.* **34,** 1258 (1940).

[150] S. S. Cohen, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 35 (1947).

[151] G. R. Wyatt and S. S. Cohen, *Biochem. J.* **55,** 774 (1953).

[152] A. Siegel and S. J. Singer, *Biochim. et Biophys. Acta* **10,** 311 (1953).

[153] A. Marshak and H. J. Vogel, *J. Biol. Chem.* **189,** 597 (1951).

[154] G. R. Wyatt, *J. Gen. Physiol.* **36,** 201 (1952).

nzyme-resistant portions. [Compare Chapters 11 and 15.] An example of ne application of Procedures II and III has been given above in Section I.2.*a*.(2). IV. Dialysis against, or other treatment with, dilute alkali:[41,142,][,146] this procedure injures, as it purifies, the deoxypentose nucleic acids, nough without noticeable change in composition;[146] the pentose nucleic cids are converted quantitatively to mononucleotides.[116] [Compare Chapers 5, 11, and 16.] Examples can, in fact, be found in the literature in which, fter the application of a modified Schmidt-Thannhauser procedure,[155] the omposition of both deoxypentose and pentose nucleic acid was deternined in the same cell or cellular fraction.[156-159] V. Preferential adsorption f pentose nucleic acid on activated charcoal:[160] this procedure is very ffective, if the preparation has been freed thoroughly of protein and if the leoxypentose nucleic acid, but not the pentose nucleic acid, is highly oolymerized, as is usually the case. The adsorbed pentose nucleic acid can oe recovered by extraction of the adsorbent with aqueous phenol.[147] VI. Separation of deoxypentose and pentose nucleic acids by means of cetylrimethylammonium bromide.[147]—Procedures I, II, V, and VI permit the ecovery of pentose nucleic acid.

Impurities of low molecular weight are best removed by dialysis against vater or, preferably, physiol. saline. The removal of protein has been lescribed before (Sections III.2.*a–d*). The elimination of high-molecular contaminants, especially polysaccharides, often is not easy, since the opportunity of using a specific enzyme[74] will not frequently present itself. Unless recourse is had to the mechanical separation of the fibrous sodium or calcium deoxypentose nucleate from the usually granular polysaccharide —a form of handicraft that often is surprisingly effective—a precipitating agent for nucleic acids, specific within certain limits, may be employed, e.g., lanthanum salts,[114,161] from which the deoxypentose nucleic acid is recovered as the potassium salt by treatment with potassium oxalate, or such tervalent complex cations as hexammine cobaltic chloride.[162]

IV. Properties of Deoxypentose Nucleic Acids

1. Elementary Composition and Standards of Integrity

Deoxypentose nucleic acids are usually isolated as the sodium salts or, occasionally, as the potassium salts.[114,161] The sodium nucleate from calf thymus has been shown to include small amounts (0.01 to 0.1 %) of ma nesium.[163] When cautiously prepared, these salts are obtained in the for of white, tough strands of fibers, resembling asbestos, or as white fiber fel after the evaporation of their frozen aqueous solution in a vacuum.[164] I addition to the cation, they contain the elements C, H, N, O, and P. Th determination of C and H usually does not contribute much to the chara terization of the substances, but N and P should be measured. Nitrogen best determined by the Dumas procedure, phosphorus by the Pregl-Lie method. For routine purposes, colorimetric estimation procedures for are convenient. We employ the method of King;[165] compare also Jones *al.*[166] The sodium nucleates contain around 12% of firmly bound wat which is best determined separately by drying the specimen at 60° for hours and correcting the other analytical values for the moisture conten The organic constituents should never be estimated in heated preparation (Compare also Peacocke.[94])

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[155] G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.* **161,** 83 (1945).
[156] A. Marshak, *J. Biol. Chem.* **189,** 607 (1951).
[157] D. Elson and E. Chargaff, *Experientia* **8,** 143 (1952).
[158] D. Elson and E. Chargaff, *Phosphorus Metabolism* **2,** 329 (1952).
[159] D. Elson, T. Gustafson, and E. Chargaff, *J. Biol. Chem.* **209,** 285 (1954).
[160] S. Zamenhof and E. Chargaff, *Nature* **168,** 604 (1951).
[161] E. Vischer, S. Zamenhof, and E. Chargaff, *J. Biol. Chem.* **177,** 429 (1949).
[162] E. Chargaff and C. Green, *J. Biol. Chem.* **173,** 263 (1948).

A selection of analytical data is given in Table I. It will be seen that th N and P values and also the atomic N/P ratios are in many cases in goo agreement with the figures calculated from the distribution of purines an pyrimidines actually found in the nucleic acids of the several genera. (Se below, Section VII.) Owing to the several structural regularities in a deoxypentose nucleic acids that will be mentioned later, the N and contents are closely similar even for preparations showing wide divergence in the proportions of individual bases; they cannot be utilized for the precis definition of composition, except to indicate the degree of purity. The sam purpose is served by the determination of the deoxypentose and pentos contents which is carried out by colorimetric comparison with suitabl nucleic acid standards.[114,116] [Compare *Dische*, Chapter 9.]

The physical tests usually applied are measurements of the absorptio spectrum in the ultraviolet and of viscosity. [See Chapters 13 and 14 While there may occur slight shifts in the position of the centers of absorp tion (between 257 and 261 mμ at pH 7), the extinction at the maximum is, in my experience, almost constant. This has often been pointed out.[114,116,167] When the extinction at the maximum and at pH 7 is expressed in th most convenient way, namely, as the atomic extinction coefficient wit respect to phosphorus and designated ε(P),[41] preparations isolated cau tiously from a large variety of sources will show surprisingly little divergence

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[163] G. Jungner, *Science* **113,** 378 (1951).

[164] Lyophilized nucleic acid preparations often carry considerable static electricity and this makes difficult the accurate weighing of small samples. We use a stati eliminator containing a polonium source.

[165] E. J. King, *Biochem. J.* **26,** 292 (1932).

[166] A. S. Jones, W. A. Lee, and A. R. Peacocke, *J. Chem. Soc.*, **1951,** 623.

[167] E. Chargaff, *Federation Proc.* **10,** 654 (1951).

TABLE I

ELEMENTARY COMPOSITION AND PURITY OF SEVERAL PREPARATIONS OF SODIUM DEOXYPENTOSE NUCLEATE

Preparation No.	Source	Preparative procedure discussed in Section III	Nitrogen, %		Phosphorus, %		Atomic N/P ratio		DNA,† % of standard	PNA,† % of standard	Ref.
			Found	Calculated*	Found	Calculated*	Found	Calculated*			
1	Calf thymus	a.(1)	15.3	15.24	9.33	9.22	3.62	3.65			a
2	Calf thymus	a.(1a)	15.3	15.24	9.14	9.22	3.69	3.65	102	1.1	b
3	Calf thymus	d.(1)	13.5‡	15.24	9.3‡	9.22	3.20‡	3.65			c
4	Sheep thymus	a.(1a)	15.3	15.24	8.94	9.23	3.78	3.65	100	1.2	d
5	Pig liver	a.(1a)	15.4	15.21	9.13	9.23	3.73	3.65	105		d
6	Human liver	a.(1a)	15.4	15.16	8.94	9.24	3.80	3.62	101		d
7	Salmon sperm	a.(1a)	14.8	15.23	8.9	9.23	3.67	3.65	108	1.5	e
8	*Arbacia lixula* sperm	a.(1a)	15.2	15.17	8.90	9.24	3.78	3.62	101	2	f

* Calculated from the distribution of nitrogenous constituents found in these species. (See references d, e, and f below.)

† Based on colorimetric comparison [see Chapter 9] with purified preparations of DNA and PNA serving as reference standards. (See references 114 and 116.)

‡ These are average values. The low nitrogen figure is probably due to the Kjeldahl procedure having been used.

References

[a] J. M. Gulland, D. O. Jordan, and C. J. Threlfall, *J. Chem. Soc.*, **1947,** 1129.

[b] C. Tamm, M. E. Hodes, and E. Chargaff, *J. Biol. Chem.* **195,** 49 (1952).

[c] E. R. M. Kay, N. S. Simmons, and A. L. Dounce, *J. Am. Chem. Soc.* **74,** 1724 (1952).

[d] E. Chargaff, and R. Lipshitz, *J. Am. Chem. Soc.* **75,** 3658 (1953).

[e] E. Chargaff, R. Lipshitz, C. Green, and M. E. Hodes, *J. Biol. Chem.* **192,** 223 (1951).

[f] E. Chargaff, R. Lipshitz, and C. Green, *J. Biol. Chem.* **195,** 155 (1952).

TABLE II

Ultraviolet Absorption of Several Preparations of Sodium Deoxypento
Nucleate*

Preparation No.	Source	Maximum		Minimum		Ref.
		mμ	ε(P)	mμ	ε(P)	
1	Calf thymus	259	6600	231	2800	a
2	Calf thymus	259	6400	232	2700	b
3	Ox spleen	259	6500	231	2900	b
4	Pig thymus	259	6850			c
5	Pig spleen	260	6800			c
6	Salmon sperm	260	6700	231	4600	d
7	*Arbacia lixula* sperm	260	7300	230	3200	e
8	Yeast	260	6100	232	2500	f
9	Avian tubercle bacilli	257	6400			g

* Preparations 1 to 8 were isolated by the procedures discussed in Section III.2.a; for Preparation 9 compare Sections II.3.a.(2) and III.2.g. See also Chapter 14 and Section IV.1 of this chapter.

References

[a] C. Tamm, M. E. Hodes, and E. Chargaff, *J. Biol. Chem.* **195,** 49 (1952).
[b] E. Chargaff, E. Vischer, R. Doniger, C. Green, and F. Misani, *J. Biol. Chem.* **177,** 405 (1949).
[c] E. Chargaff and R. Lipshitz, *J. Am. Chem. Soc.* **75,** 3658 (1953).
[d] E. Chargaff, R. Lipshitz, C. Green, and M. E. Hodes, *J. Biol. Chem.* **192,** 223 (1951).
[e] E. Chargaff, R. Lipshitz, and C. Green, *J. Biol. Chem.* **195,** 155 (1952).
[f] E. Chargaff and S. Zamenhof, *J. Biol. Chem.* **173,** 327 (1948).
[g] E. Vischer, S. Zamenhof, and E. Chargaff, *J. Biol. Chem.* **177,** 429 (1949).

from the value of ε(P) = 6600. In eight preparations of sodium deoxy ribonucleate from ox tissues (seven from thymus, one from spleen) the absorption maximum was at 259 mμ and ε(P) = 6650 with a standard error of ±50.[111] A selection of data is presented in Table II. In contrast to a statement in the literature,[101] I am inclined to consider an ε(P) higher than about 7200 as a sign of denaturation. (Compare the discussion of the general problem of hyperchromic effects by Magasanik and Chargaff[42] and in Chapter 14, and also Thomas.[168,169])

While the absence of high viscosity indicates denaturation or degradation of the sodium nucleate, no standard values can as yet be given for the viscosity of carefully prepared specimens. [Compare the discussion by *Jordan*, Chapter 13.]

Surprisingly little is known about the optical activity of intact nucleic acids. To indicate the order of magnitude, some measurements by Tamm *et al.*[170] may be quoted. With a preparation of calf thymus sodium deoxy-

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[168] R. Thomas, *Bull. soc. chim. biol.* **35,** 609 (1953).
[169] R. Thomas, *Biochim. et Biophys. Acta* **14,** 231 (1954).
[170] C. Tamm, M. E. Hodes, and E. Chargaff, *J. Biol. Chem.* **195,** 49 (1952).

ibonucleate (similar to Preparation 2 in Table I) the following values were ound for approximately 0.1 % solutions in 0.1 M phosphate buffer of pH 7.1: $[\alpha]_{\text{D}}^{25} = +100° \pm 10°$; $[\alpha]_{\text{D}}^{25}(\text{P}) = +1350° \pm 100°$; $[M]_{25}^{\text{D}}(\text{P}) = +420° \pm 25°$. $[\alpha]_{\text{D}}(\text{P})$ is the specific rotation with respect to phosphorus, equaling 100 $\alpha_{\text{D}}/bc(\text{P})$, in which b is the layer thickness and $c(\text{P})$ the phosphorus oncentration in grams per 100 cc. of solution. $[M]_{\text{D}}(\text{P})$ is the atomic rotation with respect to phosphorus, equaling $0.3098[\alpha]_{\text{D}}(\text{P})$. These terms, and the corresponding viscosity expression $\eta_{\text{sp.}}(\text{P})$ denoting the specific viscosity divided by the molarity of the solution with respect to P,[10] were adopted for the same reasons that led to the introduction of the term for extinction $\epsilon(\text{P})$.

The formation of insoluble deoxypentose nucleates by tervalent cations, especially lanthanum, has often been investigated;[23,30,114,161,171,172] but there is little information concerning other salts or metal complexes, nor do detailed solubility studies of these compounds seem to have been carried out. Complexes and precipitates with streptomycin have been described,[173,174] as has also the interaction of deoxypentose nucleic acids with dyes.[175-177]

The physical properties of the nucleic acids are outlined in Chapters 13 and 14. Estimates of the molecular weight of calf thymus deoxyribonucleic acid vary, less with the specimen than with the method of determination, from 820,000[178] to 7,700,000.[179] Mention should be made here of investigations on the electron microscopy of sodium deoxyribonucleates.[180-182]

2. DENATURATION AND DEGRADATION

A mild, but persistent, mistreatment of a protein leads to a state of malaise known, vaguely, as denaturation. It is not astonishing that the nucleic acids, especially the deoxypentose nucleic acids, which in the gradual recognition of their complex properties have emulated the proteins in many respects, have come also into this legacy. The decay of a macromolecule of a specific and complicated structure will usually go through a number of successive stages; the changes, almost imperceptible in the beginning, multiply cumulatively, until the collapse makes itself known with almost

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[171] E. Hammarsten, G. Hammarsten, and T. Teorell, *Acta Med. Scand.* **68,** 219 (1928).

[172] K. G. Stern and M. A. Steinberg, *Biochim. et Biophys. Acta* **11,** 553 (1953).

[173] S. S. Cohen, *J. Biol. Chem.* **168,** 511 (1947).

[174] H. v. Euler and L. Heller, *Arkiv. Kemi, Mineral. Geol.* **26A,** No. 14 (1948).

[175] L. F. Cavalieri and A. Angelos, *J. Am. Chem. Soc.* **72,** 4686 (1950).

[176] L. F. Cavalieri, A. Angelos, and M. E. Balis, *J. Am. Chem. Soc.* **73,** 4902 (1951).

[177] J. L. Irvin and E. M. Irvin, *J. Biol. Chem.* **206,** 39 (1954).

[178] R. Cecil and A. G. Ogston, *J. Chem. Soc.* **1948,** 1382.

[179] M. E. Reichmann, R. Varin, and P. Doty, *J. Am. Chem. Soc.* **74,** 3203 (1952).

[180] J. F. Scott, *Biochim. et Biophys. Acta* **2,** 1 (1948).

[181] R. C. Williams, *Biochim. et Biophys. Acta* **9,** 237 (1952).

[182] H. Kahler and B. J. Lloyd, Jr., *Biochim. et Biophys. Acta* **10,** 355 (1953).

explosive suddenness. The sequence leads probably from the rupture secondary valence bonds to the fission of covalent links; but the sense ma be opposite under circumstances, especially during enzymic attack: th cleavage of covalent linkages could bring about the automatic snapping c for instance, hydrogen bonds. The line separating a denaturation produ from a degradation product is not clearly drawn; but one could define denaturation products those substances whose preparation caused inte ference with the physical properties, but not with the chemical compositio of the parent nucleic acid, while the latter change will form part of th description of a degradation product.[183]

Though proteins and nucleic acids share many features, there is on essential distinction: the ideal "monomer" of a protein consists of on molecular species, the amino acid; the ideal "monomer" of a nucleic aci the nucleotide, is composed of three, namely, base, sugar, phosphoric aci This brings about multiple possibilities of breakdown. The deoxypentos nucleic acid chain can be degraded vertically, as it were, and horizontally i.e., perpendicularly to the long fiber axis and parallel to it. I shall retur to this point in the next section.

The denaturation of deoxypentose nucleic acids can be followed by tw or perhaps three, different methods: (a) modifications in viscosity behavior (b) spectral changes; and finally, but only in a very limited number o cases, (c) loss of transforming activity.[91] The second procedure, viz., spec troscopy, appears, at least at present, the most fruitful.

As regards viscosity changes induced by acid or alkali, there exists a extensive literature which cannot be reviewed here in detail [compare *Jor dan*, Chapter 13], though a few investigations should be cited.[30,90,99,184-18 But viscosity is a treacherous guide. On the one hand it can be shown tha a solution of the sodium deoxyribonucleate of calf thymus in 0.05 *M* NaCl when adjusted to pH 3 by the careful addition of acid, shows a drop o specific viscosity from 21.4 to 0.5; that the high viscosity of the solutio is regained upon neutralization within 30 minutes; but that under thes conditions an artifact is produced which, in contrast to the undenature preparation, has a highly thixotropic character.[90] On the other hand, the adjustment of similar solutions to pH 2.6 by dialysis did not affect the molecular weight (7,700,000), as determined by light scattering.[189] For a discussion of the irreversible changes accompanying the titration of nucleic acids Chapter 13 should be consulted.

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[183] C. Tamm, H. S. Shapiro, and E. Chargaff, *J. Biol. Chem.* **199,** 313 (1952).

[184] C. F. Vilbrandt and H. G. Tennent, *J. Am. Chem. Soc.* **65,** 1806 (1943).

[185] J. M. Gulland, D. O. Jordan, and H. F. W. Taylor, *J. Chem. Soc.* **1947,** 1131.

[186] J. M. Creeth, J. M. Gulland, and D. O. Jordan, *J. Chem. Soc.* **1947,** 1141.

[187] H. v. Euler and A. Fonó, *Arkiv Kemi, Mineral. Geol.* **25A,** No. 3 (1947).

[188] H. Schwander, *Helv. Chim. Acta* **32,** 2510 (1949).

[189] M. E. Reichmann, B. H. Bunce, and P. Doty, *J. Polymer Sci.* **10,** 109 (1953).

The observations of the spectral changes attending the denaturation and subsequent degradation of the nucleic acids start from the fact that the extinction of intact preparations is lower than would correspond to the sum of their constituent mononucleotides. The hyperchromic effect of degradation has been discussed for pentose nucleic acids by Magasanik and Chargaff.[12] [Compare Chapters 11 and 14.] As regards deoxypentose nucleic acids, it was Kunitz[55] who first described the intensification of the extinction brought about by deoxyribonuclease. Similar effects produced by acid, alkali, heat, or the addition of salts have been studied frequently,[190-193] in greatest detail by Thomas.[169] Owing to the lability of the pentose nucleic acids, the optical effects accompanying their denaturation and their degradation can hardly be separated; and in the deoxypentose nucleic acids, too, a further-reaching chemical degradation, namely, the removal of the purines, has been shown to have a hyperchromic effect.[170] But it may be concluded that for such effects to become noticeable in deoxypentose nucleic acids a relatively mild treatment affecting only secondary valence bonds is sufficient.[169]

The transforming activity of certain bacterial deoxypentose nucleic acids is discussed in Chapter 27. Reference may be made here, however, to the curious observation by McCarty that the transforming substance of *Pneumococcus* Type III is inactivated reversibly by ascorbic acid.[194]

The end results of chemical and enzymic degradation need not concern us here; they are discussed, in different contexts, in Chapters 5, 12, and 15. Certain aspects will also later be touched upon in this chapter as far as they bear on questions of composition and structure. What should be mentioned here—but it can only be done in the briefest form—is the existence of numerous studies on the degradation of deoxypentose nucleic acids by irradiation with ultraviolet light,[195-199] by treatment with X-rays,[200-205]

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[190] K. K. Tsuboi, *Biochim. et Biophys. Acta* **6,** 202 (1950).

[191] L. F. Cavalieri, *J. Am. Chem. Soc.* **74,** 1242 (1952).

[192] G. Frick, *Biochim. et Biophys. Acta* **8,** 625 (1952).

[193] E. R. Blout and A. Asadourian, *Biochim. et Biophys. Acta* **13,** 161 (1954).

[194] M. McCarty, *J. Exptl. Med.* **81,** 501 (1945).

[195] A. Hollaender, J. P. Greenstein, and W. V. Jenrette, *J. Natl. Cancer Inst.* **2,** 23 (1941).

[196] M. Errera, *Biochim. et Biophys. Acta* **8,** 30, 115 (1952).

[197] M. Seraydarian, A. Canzanelli, and D. Rapport, *Am. J. Physiol.* **172,** 42 (1953).

[198] R. Setlow and B. Doyle, *Biochim. et Biophys. Acta* **12,** 508 (1953).

[199] J. A. V. Butler and B. E. Conway, *Proc. Roy. Soc.* (*London*) **B141,** 562 (1953).

[200] B. Taylor, J. P. Greenstein, and A. Hollaender, *Arch. Biochem.* **16,** 19 (1948).

[201] M. Errera, *Bull. soc. chim. biol.* **33,** 555 (1951).

[202] B. E. Conway and J. A. V. Butler, *J. Chem. Soc.* **1952,** 834.

[203] G. Scholes and J. Weiss, *Biochem. J.* **53,** 567 (1953); **56,** 65 (1954); *Nature* **171,** 920 (1953).

[204] M. Daniels, G. Scholes, and J. Weiss, *Nature* **171,** 1153 (1953).

[205] V. L. Koenig and J. D. Perrings, *Arch. Biochem. and Biophys.* **44,** 443 (1953).

with ultrasound,[206,207] or with radiomimetic agents, such as the sulfur an nitrogen mustards.[77,208,209] Many of these studies have been considered i detail by Errera.[210] The effect of phenol and urea has also been studied.[21

V. Some Partial Degradation Products

1. General

Even if the assumption is made—and there is little justification for it, as will be shown later—that a deoxypentose nucleic acid preparation from a given cell is composed of only one molecular species, it will be readily understood that the problem of describing its fine structure still is insolvable. It will affect this difficulty very little whether we are dealing with a single polynucleotide chain or with two complementary chains holding each other in a complicated embrace and exposing identical backsides to the outer world. A nucleic acid chain must, according to the molecular weight assigned to it, be composed of 2500 to 15,000 mononucleotides of 4 or 5 or more varieties; and only through the most stultifying oversimplification could a particular sequence be predicted. It will suffice to point out that a chain consisting of 2500 nucleotides in the proportions found for the total deoxypentose nucleic acid of ox tissues (see Section VII) could exist in something like 10^{1500} sequential isomers.[4] Since the human mind does not enjoy contemplating the impossible for a long time, it either forgets, neglects, or reduces it. The latter operation results in the more modest desire, not to write the entire sequence of nucleotides, but to discern certain more general structural features, if any can be found.

A stepwise degradation of the nucleic acid chain appears to offer possibilities of distinction between different entities. That it may be carried out in two essentially different directions has been pointed out in the preceding section (IV.2). Owing to the great difference in stability of the glycosidic linkage in the purine and in the pyrimidine nucleosides (compare Chapters 5 and 9), it is possible to remove the purines preferentially from a deoxypentose nucleic acid chain by a carefully controlled acid hydrolysis. Products of this type for which the name apurinic acid has been proposed[170] will be discussed in the next section. Less well-defined substances resulting from a more vigorous breakdown have long been known as thymic acid.

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[206] S. G. Laland, W. G. Overend, and M. Stacey, *J. Chem. Soc.* **1952,** 303.

[207] I. E. El'piner and A. V. Gerasimova, *Doklady Akad. Nauk S.S.S.R.* **86,** 797 (1952); *Chem. Abstr.* **47,** 2225 (1953).

[208] D. T. Elmore, J. M. Gulland, D. O. Jordan, and H. F. W. Taylor, *Biochem. J.* **42,** 308 (1948).

[209] J. A. V. Butler, L. Gilbert, and D. W. F. James, *J. Chem. Soc.* **1952,** 3268. Compare this paper for references to other work.

[210] M. Errera, Mécanismes de l'Action des Radiations sur le Noyau Cellulaire, Les Éditions "Acta Medica Belgica," Bruxelles, 1952.

[211] B. E. Conway and J. A. V. Butler, *J. Chem. Soc.* **1952,** 3075.

The controlled action of deoxyribonucleases leads to breakdown products of an entirely different character, namely, to a mixture of large oligonucleotides, more resistant to enzymic action than the bulk of the nucleic acid molecule and originally designated as the "core."[212] This name has given rise to some criticism; but, since it seems to be more often used by its critics than by its proponents, it may at least have the virtue of terseness.[213] Other possible designations would be "limit polynucleotide" or "enzyme-resistant residue."

2. Preferential Removal of Purines

a. Thymic Acid

The action of dilute mineral acid at 80° or 100° on deoxypentose nucleic acid yields a rather ill-defined degradation product which, as its original discoverers believed it to contain only thymine, was designated "thymic acid."[214] It was later, however, shown to contain also cytosine.[215,216] Products of this type, often of varying, always of incompletely known, composition have played an important part in discussions of the structure of deoxypentose nucleic acid.[1,185,215-220] Their role in the nucleal reaction of Feulgen also has often been considered. [Compare *Swift*, Chapter 17.] In the course of their studies on partial degradation products and on the formation of apurinic acid Tamm *et al.*[170,183,221] prepared and analyzed a series of partial degradation products and compared their composition with that of the parent calf thymus sodium deoxyribonucleate. Their studies should be consulted for details. The rate of dialysis of the liberated purines and the composition of the dialysate, when calf thymus nucleic acid is exposed to the conditions leading to the formation of apurinic acid (pH 1.6, 37°), are shown in Fig. 2.

b. Apurinic Acid

A typical preparation of apurinic acid from the sodium deoxyribonucleate of calf thymus will be described here.

(*1*) *Preparation.*[170] To a solution of 105.0 mg. of the sodium nucleate (lyophilized, moisture content 13%) in 42.5 cc. of water a total of 12.5 cc. of 0.1 N aqueous HCl was

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[212] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **178,** 531 (1949).

[213] CORE: "A central part of different character from that which surrounds it." (Oxford English Dictionary, Vol. II, p. 990.)

[214] A. Kossel and A. Neumann, *Ber.* **26,** 2753 (1893); *Z. physiol. Chem.* **22,** 74 (1896–97).

[215] H. Steudel and P. Brigl, *Z. physiol. Chem.* **70,** 398 (1910–11).

[216] R. Feulgen, *Z. physiol. Chem.* **101,** 296 (1917-18).

[217] H. Steudel and E. Peiser, *Z. physiol. Chem.* **111,** 297 (1920).

[218] S. J. Thannhauser and B. Ottenstein, *Z. physiol. Chem.* **114,** 39 (1921).

[219] H. Bredereck and G. Müller, *Ber.* **72,** 115 (1939).

[220] J. M. Gulland, *in* Nucleic acid, *Symposia Soc. Exptl. Biol.* **1,** 1 (1947).

[221] C. Tamm, H. S. Shapiro, R. Lipshitz, and E. Chargaff, *J. Biol. Chem.* **203,** 673 (1953).

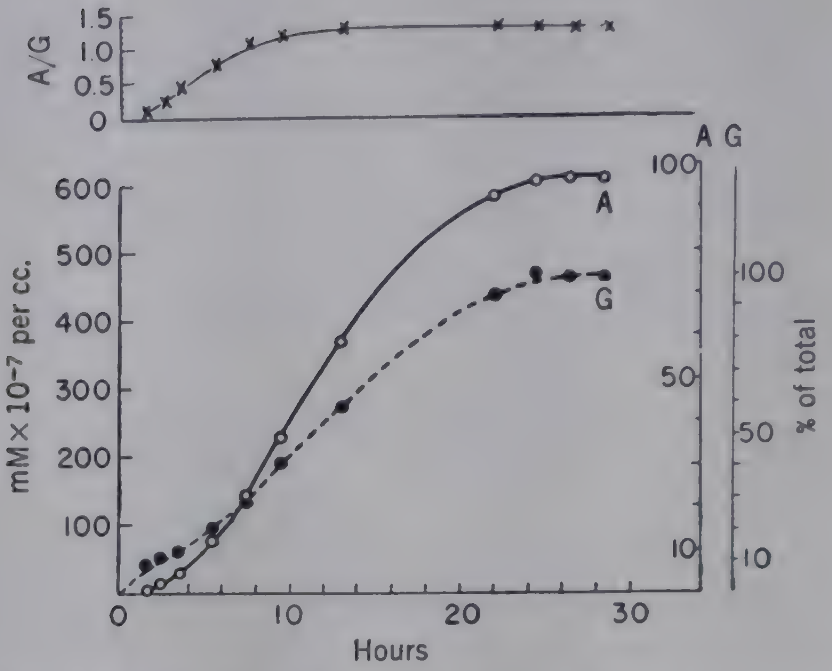

FIG. 2. Liberation of free purines from the sodium deoxyribonucleate of calf thymus at 37° and pH 1.6. The duration of the reaction is plotted as the abscissa against the concentration of adenine (A) and guanine (G) in the dialysate (left ordinate) and against the quantities of liberated purines as per cent of total adenine and guanine contained in the starting material (right ordinates). The upper part of the graph indicates the molar ratio of adenine to guanine in the dialysate. (Taken from Tamm *et al.*[170])

added gradually, when a pH of 1.6 was reached. During the addition a heavy precipitate formed in the viscous solution. The mixture was immediately transferred to a cellophane bag and dialyzed at 37° against 440 cc. of dilute HCl of pH 1.6 for 26 hours.[222] No change in pH occurred during this time. The clear inside fluid was dialyzed against 750 cc. of 0.2 *M* borate buffer (pH 7.3) for 22 hours at 4°, against running tap water (about 12°) for the same period, against frequent changes of distilled water at 4° for 24 hours, and evaporated in the frozen state in a vacuum. The apurinic acid formed a pure white fluff, weighing 76.7 mg. and containing 9.7% of moisture. It was very hygroscopic when completely dehydrated and was easily soluble in water to give a solution of about pH 6.5. The yield corresponded to 94% of the starting material, when allowance was made for the loss of purines amounting to about 20% of the initial weight.

(*2*) *Properties.* Information about two typical preparations of apurinic acid is provided in Table III. It will be seen that the recovery of the pyrimidines and, even more significantly, of the phosphorus is almost quantitative. The comparison with the data given below in Section VII will show that no distortion of the interpyrimidine ratios characteristic of the parent nucleic acid has taken place. All this is not true of thymic acid preparations. In apurinic acid the nucleotide sequence of the parent substance presumably is preserved, except that the positions of the purine

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[222] When larger quantities of apurinic acid are to be prepared, it is not necessary to carry out the acid treatment under conditions of dialysis. The subsequent neutralization and dialysis are, of course, required.

TABLE III

APURINIC ACID FROM CALF THYMUS SODIUM DEOXYRIBONUCLEATE*

	Preparation No. 1	Preparation No. 2
Nitrogen, %	6.0	6.8
Phosphorus, %	10.7	10.6
Color yield with diphenylamine as % of standard DNA†	108	95
Moles per 100 g.-atoms of P‡		
Adenine	0	0
Guanine	0	0
Total purines	0	0
Thymine	27	27
Cytosine	20	20
Total pyrimidines	47	47
Molar ratios		
Thymine to cytosine	1.3	1.3
Phosphorus to total bases	2.1	2.1
Balances, % of DNA constituent recovered in product		
Thymine	89	100
Cytosine	89	101
Phosphorus	89	96

* The figures refer to the dry preparations.

† The values are corrected for the loss in weight due to the removal of purines.

‡ When large amounts of hydrolysate are analyzed, between 1.2 and 1.3 mole % of 5-methylcytosine is found and minute residues of adenine (0.5–1 mole %) and guanine (1 mole %).

nucleotides now are occupied by nonglycosidic deoxyribose phosphate units which react as free aldehydes. When comparison is made on the basis of P content, the color yield of the reaction of apurinic acid with diphenylamine is almost identical with that given by deoxypentose nucleates.[223] [Compare *Dische*, Chapter 9.]

The absorption spectra of the sodium salt of apurinic acid and of calf thymus sodium deoxyribonucleate are compared in Fig. 3. The absorption maximum is at 267 to 268 mμ with an $\epsilon(P)$ of 4600 to 4800, the minimum

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[223] A previous statement[170, 183] that apurinic acid gives a greater color yield with diphenylamine than the corresponding quantity of deoxyribonucleic acid is incorrect. It was due to an error in the computation of a conversion factor.

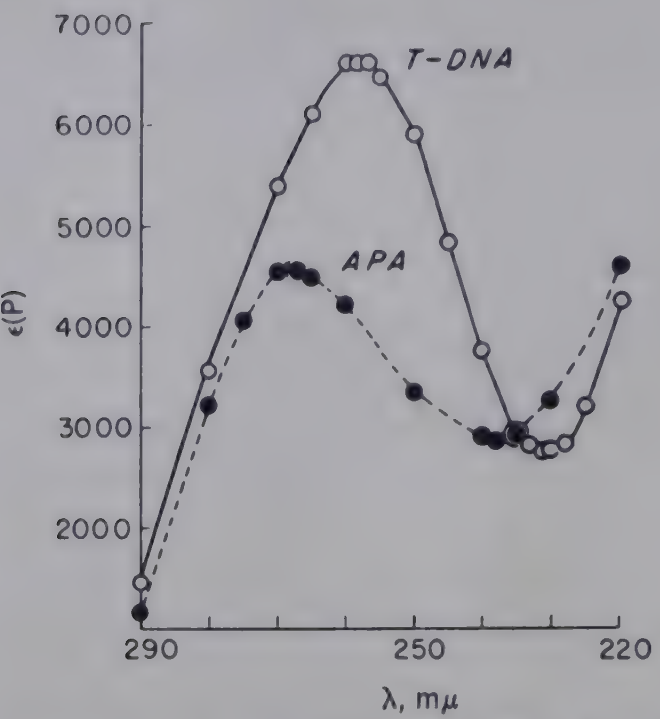

FIG. 3. Absorption spectra in the ultraviolet of sodium deoxyribonucleate of calf thymus (T-DNA) and of the sodium salt of the corresponding apurinic acid (APA) in phosphate buffer of pH 7.1. (Taken from Tamm *et al.*[170])

at 238 mμ with an ε(P) of 2900.[170,221] When the extinction coefficient is computed with respect to nucleotide P rather than to total P, the corresponding ε(NP) at the maximum is found at 9800 to 10,400: another example of the hyperchromic effect of degradation mentioned above in Section IV. The specific rotation $[\alpha]_D^{25}$ is +50°, the atomic rotation with respect to phosphorus $[M]_D^{25}(P)$ is +140°. (Compare Section IV.1.)

Other physical and chemical properties of apurinic acid and its derivatives have been discussed by Tamm and Chargaff.[224] In contrast to deoxypentose nucleates, apurinic acid reduces Fehling's or Benedict's solution on warming and reacts with ammoniacal silver solution. It gives a strong Schiff test with reduced fuchsin at room temperature. It consumes one mole of potassium bisulfite per mole of nonglycosidic sugar phosphate and is stable toward periodic acid. It yields an oxime, a 2,4-dinitrophenylhydrazone, and a benzyl mercaptal which is cleaved by alkali.

Apurinic acid preparations may be obtained from a large variety of different deoxypentose nucleic acids. They have proved convenient intermediates in the search for trace pyrimidines in nucleic acids.[104,151,170] In addition, two points should be stressed. (a) Apurinic acid, while still a polynucleotide, has an average molecular weight (estimated at 15,000[224]) that is much smaller than that of the nucleic acid from which it is prepared. (b) Apurinic acid is, in its composition, characteristic of the particular deoxypentose nucleic acid that served as the starting material. The use of preparations of this type in structural studies will be considered later.

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[224] C. Tamm and E. Chargaff, *J. Biol. Chem.* **203,** 689 (1953).

3. "CORES" (LIMIT POLYNUCLEOTIDES)

When a deoxyribonuclease acts on a deoxyribonucleic acid, we are dealing with an enzyme of as yet unrecognized specificity attacking a polynucleotide chain of as yet unknown sequence. It is a reaction whose study is likely to spread more darkness than light. Moreover, the fact that deoxyribonuclease is a phosphodiesterase of a closely circumscribed, as yet undefinable, specificity[225] producing only a very small quantity of the ultimate "monomers," the mononucleotides, in addition to a complex mixture ranging from polynucleotides to dinucleotides,[226] raises a problem of a special kind. The enzymic attack on a substrate of the complexity of a deoxypentose nucleic acid, which results in its partial cleavage, must go through an intricate pattern: every break of the original molecule produces substrates that are new and different; the enzyme must deal with kaleidoscopic substrate changes. The attempt to solve this gigantic puzzle by fitting the innumerable fragments into a plausible sequence is doomed to failure. Unless, however, deoxyribonuclease is specific only for the size, and not the quality, of the oligonucleotide fragments which it is able to cleave, in which case the first few random events would decide all subsequent ones, the order in which different pieces are detached, and the composition of those that are left behind, may serve as means of distinction between different nucleic acids.

The study of the action of crystalline pancreatic deoxyribonuclease [compare *Schmidt*, Chapter 15] on calf thymus nucleic acid has, in fact, shown a characteristic trend in the composition of dialyzable digestion products and the existence of polynucleotides, distinguished by greater resistance to enzymic attack and characterized by greatly increased ratios of adenine to guanine, thymine to cytosine, and purines to pyrimidines.[212,227] Similar studies have been carried out with nucleic acids from other sources.[119,134,228-230] It may also be mentioned that the "cores" produced by the action of two different deoxyribonucleases, those of pancreas and of germinating barley, on the same nucleic acid differ markedly in their composition.[231]

VI. Constituents of Deoxypentose Nucleic Acids

The chemistry of the ultimate breakdown products of the nucleic acids, namely, the sugars and nitrogenous constituents, is discussed in Chapters 2 and 3; the nucleosides and nucleotides are considered in Chapter 4. In

[225] C. Tamm and E. Chargaff, *Nature* **168,** 916 (1951).
[226] R. L. Sinsheimer, *J. Biol. Chem.* **208,** 445 (1954).
[227] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **187,** 1 (1950).
[228] W. G. Overend and M. Webb, *J. Chem. Soc.* **1950,** 2746.
[229] K. Moldave and C. Heidelberger, *J. Am. Chem. Soc.* **76,** 679 (1954).
[230] L. L. Uzman and C. Desoer, *Arch. Biochem. and Biophys.* **48,** 63 (1954).
[231] G. Brawerman and E. Chargaff, *J. Biol. Chem.* **210,** 445 (1954).

this brief section I shall limit myself to those aspects that are of importanc for the succeeding discussion of composition and structure.

1. Sugar

As regards the type of sugar occurring in deoxypentose nucleic acids very much has been taken for granted owing no doubt to the great difficulty of isolating the free deoxysugars. Only for the nucleosides and nucleotides obtainable from thymus nucleic acid, and strictly speaking, only for the purine derivatives, is the chemical evidence decisive. There exist, however, no indications that of the possible 2-deoxypentoses, 2-deoxyribose and 2-deoxyxylose, any but 2-deoxy-D-ribose occur in nucleic acids, though proof by actual isolation has been scanty (see Chapter 2); nor has an authentic case of the coexistence of both pentoses and deoxypentoses in the same nucleic acid been described. The lack of information on the optical activity of nucleosides and nucleotides isolated from other nucleic acids than that of calf thymus is regrettable.

The chromatographic investigation of the sugars present in different deoxypentose nucleic acids, or at any rate in their purine nucleotide moieties, has, however, been pursued on a quite extensive scale in the past few years. It is based on the enzymic degradation of the nucleic acid to the nucleoside stage, on the release of the sugar by controlled heating of the nucleosides at pH 1.5, and the chromatographic comparison, in at least three different solvent systems, of the unknown sugar with 2-deoxyribose liberated under identical conditions from calf thymus deoxyribonucleic acid.[114,232] In all instances examined heretofore, the sugars were identical and agreed in their chromatographic behavior with that of 2-deoxyribose. This tentative identification has been performed with deoxypentose nucleic acid preparations from the following sources: ox (thymus, spleen),[114] sheep (thymus, liver),[111] pig (thymus, liver),[111] man (thymus, liver, carcinoma),[111] salmon sperm,[57] sperm from four sea urchin genera,[104] yeast, and avian tubercle bacilli.[161] 2-Deoxyribose has also been demonstrated in apurinic acid.[224]

2. Nitrogenous Constituents

With very few exceptions, the bulk of the nitrogenous constituents (about 98 to 99%) of all deoxypentose nucleic acids is composed of two purines adenine and guanine, and two pyrimidines, cytosine and thymine. The principal exceptions are the deoxypentose nucleic acids of the bacteriophages T2, T4, and T6 of *E. coli*, in which 5-hydroxymethylcytosine takes the place of cytosine,[151,233] and the nucleic acid of wheat germ, in which

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[232] E. Chargaff, C. Levine, and C. Green, *J. Biol. Chem.* **175,** 67 (1948).

[233] G. R. Wyatt and S. S. Cohen, *Nature* **170,** 1072 (1952).

about one-quarter of the cytosine is replaced by 5-methylcytosine.[119,123,134] The latter has been recognized by Wyatt[123,234,235] as occurring in traces in several deoxypentose nucleic acids of animal origin (compare also reports of other investigators[104,119,170]), after an older claim of its being a constituent of the nucleic acid of tubercle bacilli[236] could not be confirmed.[161] The chemistry of these purines and pyrimidines is discussed in Chapter 3 and that of the corresponding nucleosides and nucleotides in Chapter 4. Certain bacteria and bacteriophages appear to be able to incorporate, from the medium, unusual pyrimidines, such as 5-bromouracil and 5-iodouracil, in their deoxypentose nucleic acids in replacement of part of the thymine;[237,238] but these substances can hardly qualify as normal constituents.

3. UNIDENTIFIED CONSTITUENTS

There is little sense in attempting a catalogue of our ignorance. As will appear later, most of the nitrogen and phosphorus of those deoxypentose nucleic acids that have been investigated in detail has been accounted for; and the remaining 1 to 3% may well be attributed to analytical imperfections. But only very few nucleic acids have been studied at all, and even they may contain as yet unrecognized nucleotide satellites. What must be avoided at the present stage is the grand sweep of induction that extracts general laws from single observations on ill-defined, and sometimes not even specified, preparations (often known as "commercial sperm nucleic acid").

It is not impossible that a careful search for novel nucleosides or nucleotides by means of ion-exchange chromatography (see Chapter 6) would be more rewarding than the separation of the free nitrogenous constituents by chromatography on filter paper (see Chapter 7). Studies of this type have been carried out on a few deoxypentose nucleic acids;[239-241] they have yielded no unexpected results. There exists, however, a body of analytical observations concerning differences in the hydrolysis behavior of different nucleic acids that may provide some clues.[111,114,134,161,231] The most interesting observation, perhaps, concerns the behavior of the adenine nucleotides of the deoxypentose nucleic acid of wheat germ.[134] As judged from the extent of its liberation by various hydrolyzing agents, adenine seems to occur in two types of linkage. The predominant type is broken with equal

[234] G. R. Wyatt, *Nature* **166,** 237 (1950).

[235] G. R. Wyatt, *Biochem. J.* **48,** 581 (1951).

[236] T. B. Johnson and R. D. Coghill, *J. Am. Chem. Soc.* **47,** 2838 (1925).

[237] F. Weygand, A. Wacker, and H. Dellweg, *Z. Naturforsch.* **7b,** 19 (1952).

[238] D. B. Dunn and J. D. Smith, *Biochem. J.*, **58,** X (1954).

[239] E. Volkin, J. X. Khym, and W. E. Cohn, *J. Am. Chem. Soc.* **73,** 1533 (1951).

[240] R. L. Sinsheimer and J. F. Koerner, *J. Biol. Chem.* **198,** 293 (1952).

[241] R. O. Hurst, A. M. Marko, and G. C. Butler, *J. Biol. Chem.* **204,** 847 (1953).

TABLE IV

Liberation of Adenine from Sodium Deoxypentose Nucleate of Wheat Germ and from Enzymically Produced "Cores"

Hydrolysis conditions	Moles per 100 g.-atoms P		
	Intact DNA	19% "core"	8% "core"
N H_2SO_4, 100°, 1 hr.	26.5	31.2	31.6
98% HCOOH, 175°, 2 hr.	26.3	33.3	35.4
7.5 N $HClO_4$, 100°, 1 hr.		33.2	

ease by N H_2SO_4, formic acid,[242] and perchloric acid;[153] the other type, the concentration of which is considerably increased in the nondiffusible "cores" (see Section V.3), appears resistant to sulfuric acid, but not to the other agents (see Table IV). In other words, a small proportion of the total adenylic acid contained in this nucleic acid shows the resistance to cleavage by mineral acid at 100° that is characteristic of the pyrimidine nucleotides. Similar, though not equally striking, observations have been made in the other instances cited above. It is not probable that they have to do with the type of phosphate bridges linking the nucleosides in the deoxypentose nucleic acids which presumably are 3′:5′ in most cases, though exceptions may occur. [See Chapter 12.] This stability is more likely connected with the glycosidic bond (e.g., sugar at position 1 or 3 of the purine). But this is, for the moment, only speculation.

VII. Composition of Deoxypentose Nucleic Acids

1. General

The study of the constitution and structure of a complicated cell constituent of a high molecular weight, such as a protein, a polysaccharide or a nucleic acid, goes through several well-defined stages. First, the substance must be properly recognized and also distinguished from other compounds with which it may share some, but not the decisive, properties. Next, all its ultimate organic constituents—and it may comprise one variety or many—have to be described in a qualitative fashion. As regards the nucleic acids, no better account of these two stages will be found than that given by Levene.[1] But when quantity has to be translated into quality, when discrimination has to be made not between substances that are palpably different, but between those that are seemingly identical, the task becomes difficult, dull, controversial. The center begins to be surrounded by enormous analytical slums: it is a world of decimals.

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[242] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176**, 715 (1948).

The problem of the recognition of identity and diversity in high-molecular cell constituents has been discussed before.[4] Here, it will suffice to point out that a chemical comparison with respect to identity or difference must be based on the nature and the proportions of the constituents, on the sequence in which these constituents are arranged in the molecule, and on the type and the position of the linkages that hold them together. The smaller the number of components of such a macromolecule is, the greater is the difficulty of a decision. Deviations of the analytical results from simple, integral proportions, of no importance for substances of small size, become very significant when applying to compounds whose molecular weights range in the millions; and variations in the proportions of their several constituents often will provide the only proof of the occurrence of different compounds.

The existence of significant chemical differences between deoxypentose nucleic acids of different cellular origin, or what has been called the chemical specificity of nucleic acids,[4] was discovered only a few years ago.[114] Soon after, it was possible to formulate most of the regularities that are recognized today.[4,167,243] The steps that permitted this relatively very rapid progress were the following. After the development of partition chromatography on filter paper and its qualitative application to amino acids[244] it became obvious that the high and specific absorption in the ultraviolet of the purines and pyrimidines could form the basis of a quantitative ultramicromethod, if proper procedures for the hydrolysis of the nucleic acids and for the complete separation of the hydrolysis products could be found. Such procedures were indeed developed,[245,246] and the elaboration of detailed methods[246-248] was soon followed by their application to the analysis of several nucleic acids.[114,161,242] The description of an arrangement permitting the easy demonstration of the purine and pyrimidine spots[249] and the application of a similar, commercially available, ultraviolet lamp[250] facilitated the performance of analyses. A photographic method useful for the preservation of permanent records was also described.[251,252]

[243] E. Chargaff, *J. Cellular Comp. Physiol.* **38,** *Suppl.* **1,** 41 (1951).

[244] R. Consden, A. H. Gordon, and A. J. P. Martin, *Biochem. J.* **38,** 224 (1944).

[245] E. Vischer and E. Chargaff, *J. Biol. Chem.* **168,** 781 (1947).

[246] E. Vischer and E. Chargaff, *Federation Proc.* **7,** 197 (1948).

[247] R. D. Hotchkiss, *J. Biol. Chem.* **175,** 315 (1948).

[248] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176,** 703 (1948).

[249] E. R. Holiday and E. A. Johnson, *Nature* **163,** 216 (1949).

[250] E. Chargaff, B. Magasanik, R. Doniger, and E. Vischer, *J. Am. Chem. Soc.* **71,** 1513 (1949).

[251] R. Markham and J. D. Smith, *Nature* **163,** 250 (1949).

[252] R. Markham and J. D. Smith, *Biochem. J.* **45,** 294 (1949).

2. Procedures

The principal analytical methods make use of chromatography of the nitrogenous constituents on filter paper; they are discussed in Chapter 7. For the subsequent spectrophotometric operations Chapter 14 should also be consulted. In addition, very occasional use has been made of the chromatography of the free bases on a starch column[115] and of the separation of enzymically liberated mononucleotides on ion exchangers.[240, 241] [Compare Chapter 6.] In contrast to their usefulness in other aspects, color reactions have found little direct application in studies on composition. [Compare Chapter 9.] The isotope dilution method does not appear to have been much used either.[253]

Requirements with respect to the purity of the specimens have been discussed in Sections III and IV. The most troublesome impurity is pentose nucleic acid. The analytical results on preparations containing more than 3% of this contaminant command little confidence; for careful comparisons this impurity should be reduced to less than 1.5%. Its removal has been discussed in Section III.2.*h*. Proteins and polysaccharides interfere less, but must be eliminated to an extent permitting the nucleic acid specimen to have a P content of more than 8%.

The procedures used in the laboratory of this writer and some of the modifications introduced in the course of time have been described in several publications cited before.[57,111,114,248]

3. Distribution of Purines and Pyrimidines

a. Grouping of Deoxypentose Nucleic Acids and Presentation of Results

When the composition of many specimens of deoxypentose nucleic acid from different cellular sources is compared, a very striking feature emerges, as was pointed out some years ago: two principal groups can be distinguished, namely the "AT type," in which adenine and thymine predominate, and the "GC type," in which guanine and cytosine are the major constituents.[142,167] In addition, an intermediate group was discovered in *E. coli* which is characterized by the presence of almost equimolar proportions of the four components[141,146,167] All total deoxypentose nucleic acid preparations from animal sources described up to this time belong to the AT type, which seems to be much more frequent than the others. The GC type has been encountered in several microorganisms and in some insect viruses. Consequently, the molar ratios of adenine to guanine, of thymine to cytosine, and of adenine + thymine to guanine + cytosine will be above 1 in the nucleic acids of the AT type and below 1 in those of the GC type. This will appear with particular clarity when the composition of microbial deoxypentose nucleic acids is compared; in Table XII, which will be discussed later, Nos. 1–4 are AT, Nos. 5–9 belong to the intermediate group, Nos. 10–16 to the GC type.

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[253] R. Abrams, *Arch. Biochem.* **30,** 44 (1951).

The subsequent account will demonstrate that different nucleic acid specimens of the same origin show a remarkable identity in composition; but this may be regarded, as was conjectured very early,[114] as "a statistical expression of the unchanged state of the cell," for it is possible to obtain, by the fractionation of nearly all deoxypentose nucleates, preparations of a composition representative of all three types mentioned above. (See Section VIII.)

The comparison of analytical results obtained by different workers using procedures that are almost never identical and often radically different suffers from a great deal of uncertainty. If, moreover, a large number of analyses of several well-characterized and purified preparations are reported in one instance, and, in another, single analyses of ill-described specimens, a dilemma between encyclopedic comprehensiveness and overjudicious exclusiveness will occur. For analytical results to be acceptable the following minimum requirements should be fulfilled. At least two completely characterized preparations isolated separately from two independently collected specimens of the starting material should be analyzed in at least two different hydrolysis experiments, and a full balance of recovered components on a molar basis should be drawn. It is surprising how seldom these modest wishes are met.

The compilation of data presented in Tables V to XIII comprises the major part, if not all, of the analytical results with deoxypentose nucleic acids. In general, only analyses performed on isolated nucleic acid specimens have been included, and several results had to be omitted because of lack of pertinent information. The total recovery has been indicated, whenever it was given in the literature; but, in order to facilitate comparison, all molar proportions have been corrected for a 100% recovery in terms of nucleic acid P.

b. *Composition Differences and Similarities*

After the existence of chemical differences between deoxypentose nucleic acids of different origin was first suggested,[4,114,161,254] agreement was not general. This is not surprising, as in many cases very extensive work is required, in order to prove a divergent composition. But there is little doubt at present that there exist many different deoxypentose nucleic acids and that their number may be even much larger than would be revealed by merely analytical differentiation. While, therefore, the question whether there exists more than one deoxypentose nucleic acid can be answered by

[254] E. Chargaff, S. Zamenhof, and C. Green, *Nature* **165,** 756 (1950).

TABLE V

Distribution of Purines and Pyrimidines in Deoxyribonucleic Acids from Different Organs of Four Mammalian Genera*

Proportions in moles of nitrogenous constituent per 100 g.-atoms of phosphorus in hydrolysate, corrected for a 100% recovery, with their standard errors.

Genus	Organ	No. of preparations	Adenine		Guanine		Cytosine		Thymine		Average total recovery Mole %	Ratio of adenine + thymine to guanine + cytosine
			Mole %	Std. error	Mole %	Std. error	Mole %	Std. error	Mole %	Std. error		
Ox	Thymus	21	29.0	0.2	21.2	0.2	21.2	0.2	28.5	0.2	93.3	1.36
	Liver	2	28.8	0.3	21.0	0.6	21.1	0.2	29.0	0.3	94.1	1.37
Sheep	Thymus	2	29.3	0.2	21.4	0.3	21.0	0.2	28.3	0.2	94.3	1.36
	Liver	2	29.3	0.2	20.7	0.2	20.8	0.3	29.2	0.3	89.9	1.41
Pig	Thymus	2	30.0	0.2	20.4	0.3	20.7	0.2	28.9	0.4	93.9	1.43
	Liver	2	29.4	0.3	20.5	0.4	20.5	0.3	29.7	0.4	95.3	1.44
	Spleen	1	29.6	0.3	20.4	0.9	20.8	0.2	29.2	0.7	96.0	1.43
	Thyroid	1	30.0	0.4	20.8	0.4	20.7	0.3	28.5	0.7	97.4	1.41
Man	Thymus	1	30.9	0.6	19.9	0.5	19.8	0.7	29.4	0.4	94.6	1.52
	Liver	4	30.3	0.2	19.5	0.2	19.9	0.2	30.3	0.2	95.4	1.54

* Taken from E. Chargaff and R. Lipshitz, *J. Am. Chem. Soc.* **75,** 3658 (1953).

pointing to the information collected in tabular form in this section, a decision whether different tissues of the same host yield identical or different nucleic acid specimens is more difficult and will, perhaps, not be definite before the analytical studies can be supplemented by other tests, for instance, a completely reproducible fractionation process. For the moment, it would appear that differences in the composition of preparations from different organs, if they exist at all, are of very doubtful significance, although some contrary findings have been reported following the administration of steroid sex hormones.[255]

The results of a comparative study of the composition of many preparations from different organs of ox, sheep, pig, and man[111] are summarized in Tables V and VI. In no case were significant differences in composition found when the deoxyribonucleic acids isolated from the thymus and liver of the same genus were compared statistically, nor were differences found between human normal and carcinomatous liver. In Table VI the significance of differences between different genera is considered. It will be seen

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[255] N. I. Gold and S. H. Sturgis, *J. Biol. Chem.* **206,** 51 (1954).

TABLE VI

Comparison between Deoxyribonucleic Acids from Different Mammals*

Proportions in moles of nitrogenous constituent per 100 g.-atoms of P in hydrolysate, corrected for a 100% recovery (with their standard errors in parentheses).

	Ox	Sheep	Pig	Man	Significance of differences† between DNA of					
					Ox and sheep	Ox and pig	Ox and man	Sheep and pig	Sheep and man	Pig and man
Adenine	29.0 (0.2)	29.3 (0.2)	29.8 (0.2)	30.4 (0.2)	−	+	++	−	++	±
Guanine	21.2 (0.2)	21.1 (0.3)	20.4 (0.3)	19.6 (0.3)	−	±	++	−	+	−
Cytosine	21.2 (0.2)	20.9 (0.2)	20.7 (0.2)	19.9 (0.2)	−	±	++	−	+	+
Thymine	28.7 (0.2)	28.7 (0.2)	29.1 (0.3)	30.1 (0.2)	−	−	++	−	++	+
Number of preparations	23	4	6	5						
Number of hydrolyses	33	12	19	21						

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* Compare Table V for data on individual organs.

† The following notation was adopted to indicate the degree of significance of the differences in composition, based on the statistical estimate (by the *t* method) of *P*, the probability of identity: $P < 0.1\%$, ++; $P < 1\%$, +; $P < 5\%$, ±; $P > 5\%$, −.

TABLE VII

SELECTION OF DATA ON PURINE AND PYRIMIDINE CONTENTS OF SODIUM DEOXYRIBONUCLEATE PREPARATIONS FROM CALF THYMUS

Proportions in moles of nitrogenous constituent per 100 g.-atoms of P in hydrolysate, corrected for a 100% recovery.*

No.	Adenine	Guanine	Cytosine†	5-Methyl-cytosine	Thymine	$\frac{A+T}{G+C+MC}$†	Actual recovery	Remarks‡	Ref.
1	28.0	22.0	19.9		30.1	1.35	88.4	?	a
2	27.3	22.7	21.6		28.4	1.26	93.7		a
3	27.9	23.8	19.9		28.3	1.29	98.8		b
4	27.4	22.4	20.7		29.5	1.32	97.0	?	b
5	28.0	23.5	20.4		28.1	1.28	100.2		b
6	29.2	20.8	20.8		29.2	1.40	96		c
7	28.2	21.5	21.2	1.3	27.8	1.27	90		d
8	28.9	22.2	21.1		27.8	1.31	90		e
9	29.2	21.9	21.9		27.1	1.29	96	?	e
10	29.2	20.2	21.3		29.2	1.41	89		e
11§			20.0	1.3	28.7		92		e
12	30.1	21.5	20.4		28.0	1.39	93	?	f
13	28.4	21.1	22.1		28.4	1.31	95		f
14	27.6	21.5	21.2	1.9	27.9	1.24	89.7		g
15	28.0	20.9	21.4	1.9	27.8	1.26	91.0		g
16	28.7	21.7	20.7	1.7	27.2	1.27		?	h
17	27.1	23.2	21.8		27.9	1.22	96.5		i
18	27.4	21.4	21.0	1.1	29.1	1.30	99.6	?	i

* When more than one value was reported for the same preparation, the average has been computed. In Preparations 1 and 2, the first complete analyses reported in the literature, the purines and pyrimidines were estimated in separate hydrolysates. For this reason, the total purines and the total pyrimidines were adjusted individually to a 50% recovery.

† Where no methylcytosine figures are given, the values for cytosine include methylcytosine in most cases. For the calculation of ratios cytosine and methylcytosine were taken together. The average of all ratios reported here is 1.30. A = adenine; G = guanine; C = cytosine; MC = methylcytosine; T = thymine.

‡ A question mark indicates that the total recovery was below 85 mole %, or that the ratio of adenine to thymine was below 0.95 or above 1.05. It has also been used when no recovery was reported or when other factors rendered the results doubtful.

§ This preparation was analyzed as the apurinic acid from calf thymus sodium deoxyribonucleate.

References (Unless pointed out otherwise, the analytical methods were based on filter-paper chromatography.)

[a] E. Chargaff *et al.*, *J. Biol. Chem.* **177,** 405 (1949).

[b] M. M. Daly *et al.*, *J. Gen. Physiol.* **33,** 497 (1950); chromatography of free bases on starch columns.

[c] A. Marshak and H. J. Vogel, *J. Biol. Chem.* **189,** 597 (1951).

[d] G. R. Wyatt, *Biochem. J.* **48,** 584 (1951).

[e] C. Tamm *et al.*, *J. Biol. Chem.* **195,** 49 (1952).

[f] C. Tamm *et al.*, *J. Biol. Chem.* **199,** 313 (1952).

[g] S. G. Laland *et al.*, *J. Chem. Soc.* **1952,** 3224.

[h] R. L. Sinsheimer and J. F. Koerner, *J. Biol. Chem.* **198,** 293 (1952); ion-exchange chromatography of mononucleotides.

[i] R. O. Hurst *et al.*, *J. Biol. Chem.* **204,** 847 (1953); ion-exchange chromatography of mononucleotides.

that the adenine and thymine contents increase, and the guanine and cytosine contents decrease, in the following order: ox, sheep, pig, man. The differences between two adjoining columns are, in general, not significant or of doubtful significance, when analyzed statistically; but the probability of identity becomes very small, when the specimens from bovine and human tissues are compared.

Table V includes information on the results of an analysis of 21 different preparations of calf thymus sodium deoxyribonucleate. Many individual analyses of calf thymus preparations are compiled in Table VII. It will be seen that the agreement is, on the whole, better than would have been anticipated. Table VIII collects data on other bovine tissues, Tables IX and X on the nucleic acids from mammalian and other animal sources, respectively.

The high content of 5-methylcytosine in the sodium deoxypentose nucle-

TABLE VIII

SELECTION OF DATA ON PURINE AND PYRIMIDINE CONTENTS OF SODIUM DEOXYPENTOSE NUCLEATE PREPARATIONS FROM BOVINE TISSUES

Proportions in moles of nitrogenous constituent per 100 g.-atoms of P in hydrolysate, corrected for a 100% recovery.*

No.	Tissue	Adenine	Guanine	Cytosine	5-Methylcytosine	Thymine	$\frac{A+T}{G+C+MC}$ †	Actual recovery	Remarks	Ref.
1	Spleen	27.9	22.1	20.7		29.3	1.34	87.9		[a]
2	Spleen	28.2	21.2	21.0	1.3	28.2	1.30	90		[b]
3	Spleen	28.6	20.9	20.7	1.4	28.3	1.32	88.2		[c]
4	Spleen	27.9	22.7	20.8	1.3	27.3	1.23	100		[d]
5	Spleen	27.7	22.1	21.8		28.4	1.28	98.0		[e]
6	Liver	28.8	21.0	21.1		29.0	1.37	94.1		[f]
7	Liver	27.4	22.5	21.9		28.2	1.25	95.6		[e]
8	Pancreas	27.8	21.9	21.7		28.5	1.29	97.8		[e]
9	Kidney	28.3	22.6	20.9		28.2	1.30	96.9		[g]
10	Testes	27.0	22.9	22.3		27.8	1.21	96.0		[e]
11	Sperm	28.7	22.2	20.7	1.3	27.2	1.26	90	?	[b]

* Compare footnotes in Table VII.

† The average of all ratios reported here is 1.29.

References

[a] E. Chargaff *et al.*, *J. Biol. Chem.* **177,** 405 (1949).

[b] G. R. Wyatt, *Biochem. J.* **48,** 584 (1951).

[c] S. G. Laland *et al.*, *J. Chem. Soc.* **1952,** 3224.

[d] G. R. Wyatt and S. S. Cohen, *Biochem. J.* **55,** 774 (1953).

[e] R. O. Hurst *et al.*, *J. Biol. Chem.* **204,** 847 (1953); ion-exchange chromatography of mononucleotides.

[f] E. Chargaff and R. Lipshitz, *J. Am. Chem. Soc.* **75,** 3658 (1953).

M. M. Daly *et al.*, *J. Gen. Physiol.* **33,** 497 (1950); chromatography of free bases on starch columns.

TABLE IX

Selection of Data on Purine and Pyrimidine Contents of Sodium Deoxypentose Nucleate Preparations from Different Tissues of Several Mammalian Genera

Proportions in moles of nitrogenous constituent per 100 g.-atoms of P in hydrolysate, corrected for a 100% recovery.*

No.	Genus	Tissue	Adenine	Guanine	Cytosine	Thymine	$\frac{A+T}{G+C}$	Actual recovery	Remarks	Ref
1	Mouse	Sarcoma	29.7	21.9	22.8	25.6	1.24	80	?	a
2	Rat	Bone marrow	28.6	21.4	20.4†	28.4	1.33		?	b
3	Sheep	Thymus	29.3	21.4	21.0	28.3	1.36	94.3		c
4	Sheep	Liver	29.3	20.7	20.8	29.2	1.41	89.9		c
5	Sheep	Spleen	28.0	22.3	21.1	28.6	1.30	92.9		d
6	Sheep	Sperm	28.8	22.0	21.0‡	27.2	1.27	88.5	?	b
7	Pig	Thymus	30.9	19.9	19.8	29.4	1.52	94.6		c
8	Pig	Liver	29.4	20.5	20.5	29.7	1.44	95.3		c
9	Pig	Spleen	29.6	20.4	20.8	29.2	1.43	96.0		c
10	Pig	Thyroid	30.0	20.8	20.7	28.5	1.41	97.4	?	c
11	Man	Thymus	29.8	20.2	18.2	31.8	1.60	91	?	e
12	Man	Thymus	30.9	19.9	19.8	29.4	1.52	94.6		c
13	Man	Liver	30.3	19.5	19.9	30.3	1.53	95.4		c
14	Man	Spleen	29.2	21.0	20.4	29.4	1.42	96.6		f
15	Man	Sperm	30.9	19.1	18.4	31.6	1.67	96		e
16	Man	Sperm	30.7	19.3	18.8	31.2	1.62	92		e
17	Horse	Spleen	29.6	22.9	20.1	27.5	1.33	95.7	?	d

* Compare footnotes in Table VII.

† In addition, 1.1 mole % of 5-methylcytosine was found.

‡ In addition, 1.0 mole % of 5-methylcytosine was found.

References

[a] S. G. Laland *et al.*, *J. Chem. Soc.* **1952,** 3224.

[b] G. R. Wyatt, *Biochem. J.* **48,** 584 (1951).

[c] E. Chargaff and R. Lipshitz, *J. Am. Chem. Soc.* **75,** 3658 (1953).

[d] M. M. Daly *et al.*, *J. Gen. Physiol.* **33,** 497 (1950); chromatography of free bases on starch columns.

[e] E. Chargaff *et al.*, *Nature* **165,** 756 (1950); correction as for Preparations 1 and 2 in Table VII.

[f] R. O. Hurst *et al.*, *J. Biol. Chem.* **204,** 847 (1953); ion-exchange chromatography of mononucleotides.

ate of wheat germ has been mentioned before. Data on the purine and pyrimidine composition of different preparations of this nucleic acid analyzed in four laboratories are presented in Table XI; the agreement is satisfactory. As has been mentioned before, little information on other plant nucleic acids is available.

What is known about the composition of microbial deoxypentose nucleic acids and some related compounds from rickettsiae and viruses is collected in Table XII. In view of the difficulty of securing sufficient material it is

TABLE X

Purine and Pyrimidine Contents of Sodium Deoxypentose Nucleates of Various Animals (from Birds Downward)

Proportions in moles of nitrogenous constituent per 100 g.-atoms of P in hydrolysate, corrected for a 100% recovery.*

No.	Animal	Tissue	Adenine	Guanine	Cytosine	5-Methyl-cytosine	Thymine	$\frac{A+T}{G+C+MC}$	Actual recovery	Remarks	Ref.
1	Hen	Erythrocytes	28.8	20.5	21.5		29.2	1.38	93.7		*a*
2	Hen	Erythrocytes	28.0	22.0	21.6		28.4	1.29	97.4		*b*
3	Hen	Egg white	29.7	21.5	21.3		27.5	1.34		?	*c*
4	Turtle	Erythrocytes	28.7	22.0	21.3		27.9	1.31	104.1		*a*
5	Shad	Testes	28.4	21.8	20.5		29.3	1.36	92.9		*a*
6	Herring	Testes	27.2	19.3	22.3	2.7	28.6	1.26	92.0		*d*
7	Herring	Testes	27.9	19.5	21.5	2.8	28.2	1.28	89.5		*d*
8	Herring	Sperm	27.8	22.2	20.7	1.9	27.5	1.23	91		*e*
9	Trout	Sperm	29.8	22.5	20.2		27.5	1.34	98.6	?	*a*
10	Salmon	Sperm	29.7	20.8	20.4		29.1	1.43	94.2		*f*
11	*Locusta migratoria*	Whole	29.3	20.5	20.7	0.2	29.3	1.41		?	*e*
12	*Arbacia punctulata*	Sperm	28.4	19.5	19.3		32.8	1.58	93.9	?	*a*
13	*Arbacia lixula*	Sperm	31.2	19.1	19.2		30.5	1.61	94.2		*g*
14	*Echinus esculentus*	Sperm	30.9	19.4	18.4	1.8	29.4	1.52	92		*e*
15	*Echinocardium cordatum*	Sperm	32.9	17.0	17.9		32.2	1.86	96.4		*g*
16	*Psammechinus miliaris*	Sperm	32.6	17.8	17.8		31.9	1.81	94.0		*g*
17	*Paracentrotus lividus*	Sperm	32.8	17.7	17.3	(1.1)†	32.1	1.85	94.7		*g*

* Compare footnotes in Table VII.

† Separate analyses indicate a content in 5-methylcytosine corresponding to 6.6% of the cytosine value.

References

[a] M. M. Daly *et al.*, *J. Gen. Physiol.* **33,** 497 (1950); chromatography of free bases on starch columns.
[b] R. O. Hurst *et al.*, *J. Biol. Chem.* **204,** 847 (1953); ion-exchange chromatography of mononucleotides.
[c] H. Fraenkel-Conrat and E. D. Ducay, *Biochem. J.* **49,** XXXIX (1951).
[d] S. G. Laland *et al.*, *J. Chem. Soc.* **1952,** 3224.
[e] G. R. Wyatt, *Biochem. J.* **48,** 584 (1951).
[f] E. Chargaff *et al.*, *J. Biol. Chem.* **192,** 223 (1951).
[g] E. Chargaff *et al.*, *J. Biol. Chem.* **195,** 155 (1952).

not astonishing that some of the analyses are not of desirable quality, but the trend is evident. It shows a very wide range of composition differences going from the extreme AT type (yeast) to the extreme GC type (tubercle bacilli). It is remarkable that all specimens from acid-fast bacteria (Nos.

TABLE XI

PURINE AND PYRIMIDINE CONTENTS OF SODIUM DEOXYPENTOSE NUCLEATE PREPARATIONS FROM WHEAT GERM

Proportions in moles of nitrogenous constituent per 100 g.-atoms of P in hydrolysate, corrected for a 100% recovery.*

No.	Adenine	Guanine	Cytosine	5-Methyl-cytosine	Thymine	$\frac{A+T}{G+C+MC}$	Actual re-covery	Re-marks	Ref
1	26.5	23.5	17.2	5.8	27.0	1.15	84.5		a
2	27.3	22.7	16.8	6.0	27.1	1.19	96.2		b
3	27.1	20.2	19.6	5.7	27.4	1.20	83.0	?	c
4	26.8	23.2	16.7	5.3	28.0	1.21	99.5		d

* Compare footnotes in Table VII.

References

[a] G. R. Wyatt, *Biochem. J.* **48,** 584 (1951).
[b] G. Brawerman and E. Chargaff, *J. Am. Chem. Soc.* **73,** 4052 (1951).
[c] S. G. Laland *et al., J. Chem. Soc.* **1952,** 3224.
[d] R. O. Hurst *et al., J. Biol. Chem.* **204,** 847 (1953); ion-exchange chromatography of mononucleotide

12–16 in Table XII) belong to this type. Even more remarkable, perhaps is the good agreement in the analytical results on 5 strains of *E. coli* (Nos 5–9). The composition of the T2, T4, and T6 phages, based on the work o Wyatt and Cohen,[151] is shown in Table XIII. Attention should also b drawn to a study of the composition of a series of insect viruses in which again, all three types of deoxypentose nucleic acid appear to have bee encountered.[154]

It should be mentioned that only in a few cases a statistical interpretatio of the significance of the analytical findings has been attempted.[57, 104, 111, 12 The conclusions with respect to the mammalian nucleic acids have bee mentioned before. In a similar study of the nucleic acids of four differen sea urchin genera[104] (compare also the work on the effect of these prepara tions on developing sea urchin eggs[256]) it was concluded that the difference in the composition of the preparations from *Echinocardium*, *Psammechinus* and *Paracentrotus* (Nos. 15–17 in Table X) were not sufficient to permit a distinction, but that the preparations from *Arbacia lixula* (No. 13) differed significantly from the others.

VIII. Fractionation of Deoxypentose Nucleic Acids

1. GENERAL

Some of the criteria on which a decision on the difference or identity o macromolecules of an ostensibly identical composition must be based hav

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[256] S. Hörstadius, I. J. Lorch, and E. Chargaff, *Exptl. Cell Research* **6,** 440 (1954).

TABLE XII

PURINE AND PYRIMIDINE CONTENTS OF SODIUM DEOXYPENTOSE NUCLEATE PREPARATIONS FROM MICROORGANISMS, ETC.

Proportions in moles of nitrogenous constituent per 100 g.-atoms of P in hydrolysate, corrected for a 100% recovery.*

No.	Organism	Adenine	Guanine	Cytosine	Thymine	$\frac{A+T}{G+C}$	Actual recovery	Remarks	Ref.
1	Yeast	31.7	18.3	17.4	32.6	1.80	74.8	?	*a*
2	Yeast	31.3	18.7	17.1	32.9	1.79	92.0		*b*
3	*Pneumococcus* type III	29.8	20.5	18.0	31.6	1.59	92.5	?	*c*
4	*Hemophilus influenzae* type C	31.9	18.2	19.6	30.2	1.64	92.7	?	*d*
5	*E. coli* (mutant B/r)	22.5	24.5	25.8	27.2	0.99		?	*e*
6	*E. coli* (mutant B/r)	23.3	23.6	25.6	27.6	1.03		?	*e*
7	*E. coli* (K-12)	26.0	24.9	25.2	23.9	1.00	94.9	?	*f*
8	*E. coli* (UQ)	25.6	25.0	25.5	23.9	0.98	93.7	?	*f*
9	*E. coli* (thymineless)	25.4	24.1	25.7	24.8	1.01	89.2		*f*
10	*Serratia marcescens*	20.7	27.2	31.9	20.1	0.69	87.0		*d*
11	*B. Schatz*	19.9	29.1	32.3	18.6	0.63	86.9	?	*d*
12	*Mb. tuberculosis* (human)	18.0	28.5	33.5	20.0	0.61		?	*e*
13	*Mb. tuberculosis* (human)	19.3	28.2	34.9	17.5	0.58	67.6	?	*g*
14	*Mb. tuberculosis* (bovine)	17.8	29.3	33.8	19.0	0.58		?	*e*
15	*Mb. tuberculosis* (avian)	15.1	34.9	35.4	14.6	0.42	76.3	?	*a*
16	*Mb. phlei*	18.0	31.6	34.8	15.5	0.50	79.0	?	*g*
17	*Rickettsia burneti*	29.5	22.5	22.0	26.0	1.25	96	?	*h*
18	*Rickettsia prowazeki*	35.7	17.1	15.4	31.8	2.08	96	?	*h*
19	*E. coli* phage T5	30.3	19.5	19.5	30.8	1.57	93		*i*
20	Vaccinia virus	29.5	20.6	20.0	29.9	1.46	100		*i*

* Compare footnotes in Table VII.

References

[a] E. Vischer *et al.*, *J. Biol. Chem.* **177,** 429 (1949).

[b] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **187,** 1 (1950).

[c] M. M. Daly *et al.*, *J. Gen. Physiol.* **33,** 497 (1950); chromatography of free bases on starch columns.

[d] S. Zamenhof *et al.*, *Biochim. et Biophys. Acta* **9,** 402 (1952).

[e] J. D. Smith and G. R. Wyatt, *Biochem. J.* **49,** 144 (1951).

[f] B. Gandelman *et al.*, *Biochim. et Biophys. Acta* **9,** 399 (1952).

[g] S. G. Laland *et al.*, *J. Chem. Soc.* **1952,** 3224.

[h] G. R. Wyatt and S. S. Cohen, *Nature* **170,** 846 (1952).

[i] G. R. Wyatt and S. S. Cohen, *Biochem. J.* **55,** 774 (1953).

TABLE XIII

PURINE AND PYRIMIDINE CONTENTS OF SODIUM DEOXYPENTOSE NUCLEATE PREPARATIONS FROM E. COLI PHAGES T2, T4, AND T6

Proportions in moles of nitrogenous constituent per 100 g.-atoms of P in hydrolysate, corrected for a 100% recovery.*

No.	Material	Adenine	Guanine	5-Hydroxy-methyl-cytosine	Thymine	$\frac{A+T}{G+HMC}$	Actual re-covery	Ref
1	T2r$^+$ DNA	32.5	18.2	16.7	32.6	1.86	97	[a]
2	T2r DNA	32.4	18.3	17.0	32.4	1.84	98	[a]
3	T6r$^+$ DNA	32.5	18.3	16.7	32.5	1.86	99	[a]
4	T6r$^+$ DNA	30.9	18.4	17.4	33.3	1.79	97	[b]
5	T2r$^+$ virus	32.0	18.0	16.8	33.3	1.88	99	[a]
6	T2r virus	32.3	17.6	16.7	33.4	1.91	95	[a]
7	T4r$^+$ virus	32.3	18.3	16.3	33.1	1.89	96	[a]
8	T4r virus	32.2	18.0	16.3	33.5	1.91	94	[a]
9	T6r$^+$ virus	32.5	17.8	16.3	33.5	1.93	99	[a]
10	T6r virus	32.3	17.7	16.6	33.4	1.91	88	[a]

* Compare footnotes in Table VII. HMC = 5-hydroxymethylcytosine.

References

[a] G. R. Wyatt and S. S. Cohen, *Biochem. J.* **55,** 774 (1953).
[b] C. F. Crampton *et al.*, *J. Biol. Chem.* **211**, 125 (1954).

been pointed out in the preceding section. Strictly speaking, the validity of many of the methods of classical organic chemistry ends with the mixed melting point. Beyond, it will often be a matter of preference or utility what to call identical and what different. If a compound is assigned a mainly mechanical function, its composition, let alone the sequence of its constituents, will appear of little importance. The function of a bag is to hold, of a trestle to support; what they are made of is of no consequence. The biochemical literature, especially that dealing with the proteins and polysaccharides, abounds in falsely generic terms which a profounder insight will undoubtedly resolve into many different individuals. Our science, so drunk with dynamics, is slowly learning to pay attention to the motions of the immovable.

For this reason, attention began to be paid to the question of differences only when specific biological functions were assigned to the nucleic acids. In the light of current conceptions, a preparation of deoxypentose nucleic acid, presumably an important component of the genetic material, could be regarded as consisting of many chemically different, though closely related individuals, the constant composition of the whole being a statistical reflection of the unchanging condition of the cell. That there is, in fact, little fluctuation in composition in different nucleic acid preparations of the same origin has been stressed before in connection with the results presented in Tables V and VI. But, as has been pointed out (Section V.1), the number of possible permutations within a nucleic acid chain, even without a change in composition, is truly enormous; and many fine points cannot be considered at the present state of our knowledge, as, for instance, whether two nucleic acids of the same composition, but differing in some details of sequence or in their terminal nucleotides, are to be regarded as different entities. One must, for the time being, rely on relatively massive changes in composition.

Although the possibility that a deoxypentose nucleic acid preparation from a given species represents a mixture of many different individuals has been discussed occasionally, it is only in recent times that successful fractionation experiments have been recorded. Attempts to separate highly polymerized preparations by fractional centrifugation[234] or adsorption on charcoal[160] were of no avail. Indications of heterogeneity, with respect to their metabolic origin, of deoxypentose nucleate preparations from rat tissues have been presented by Bendich *et al.*[257] The evidence rests on differences in the incorporation of isotope observed with two nucleic acid preparations differing in their solubility in physiol. saline. In the absence of analytical information it is not possible to say whether the fractions differed in composition, nor, in fact, whether they were pure nucleic acids. In the light of the experiments of Crampton *et al.*[10] on the dissociation and reassociation of nucleohistone, the material insoluble in physiol. saline probably represented a, perhaps partially degraded, protein nucleate.

A process best described as the fractional dissociation of a nucleoprotein has permitted the separation of many deoxypentose nucleate preparations into a series of fractions of divergent purine and pyrimidine contents. The procedure was first applied to calf thymus nucleohistone[258] and later extended to the fractionation of other nucleic acids through their complexes with histone,[259] globin,[259] or polylysine.[260] Subsequently, a related procedure was described in a preliminary form in which fractionation was achieved by the gradual elution of the sodium nucleate from a histone-kieselguhr column.[261]

2. Fractional Dissociation of Nucleohistone or Protein Nucleates

The experiments are based on the observation[10] that when nucleohistone, prepared as described in Section II.3.*a*.(1), is treated with chloroform in the absence of electrolytes the entire nucleic acid P is found in the resulting

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[257] A. Bendich, P. J. Russell, Jr., and G. B. Brown, *J. Biol. Chem.* **203,** 305 (1953).

[258] E. Chargaff, C. F. Crampton, and R. Lipshitz, *Nature* **172,** 289 (1953).

[259] C. F. Crampton, R. Lipshitz, and E. Chargaff, *J. Biol. Chem.*, **211,** 125 (1954).

[260] P. Spitnik, R. Lipshitz, and E. Chargaff, in preparation.

[261] G. L. Brown and M. Watson, *Nature* **172,** 339 (1953).

TABLE XIV

Dissociation of Nucleohistone in Salt Solutions, after a Single Treatment with Chloroform

NaCl molarity	Nucleic acid P in supernatant, as % of initial	
	Exp. 1	Exp. 2
0.60	39.5	42.2
0.65	52.4	45.9
0.70	58.4	58.9
0.80	78.6	79.0
1.00		94.6

gel, whereas a similar treatment in salt solutions of increasing strengt results in the proportionate detachment of increasing quantities of nuclei acid. This is illustrated in Table XIV,[8] which is based on the experiments o Crampton *et al.*[10]

For details of the procedures the original papers[258, 259] should be consulted. Full data on two abbreviated fractionation runs with calf thymu nucleohistone, in which only three fractions were collected, are given i Table XV.[259]

Nucleohistone preparation N-NH was isolated, and precipitated at 0.15 *M* NaC concentration, as described in Section II.3.*a*.(1); preparation R-NH was isolate similarly, but from a solution that had first been exposed to *M* NaCl. Solutions o these preparations in distilled water (previously adjusted to pH 7), containing abou 90 μg. P per cc., were mixed with an equal volume of 1.3 *M* NaCl solution and, afte an interval of 30 minutes, treated in a high-speed mixer with one-half volume o chloroform – octanol (9:1) for 2 minutes. From the supernatant fluids, obtained b centrifugation (15 minutes, 2000 × *g*), the sodium nucleate fractions 1-I and 2- (Table XV) were isolated as described in Section III.2.c. The sedimented gels wer reextracted, as described above, first with portions of 0.9 *M* NaCl equal to one-hal of the volume of nucleohistone solution (fractions 1-II and 2-II) and then with 2.6 NaCl (fractions 1-III and 2-III). The results are shown in Table XV. The deoxyribose content of the fractions varied from 95 to 100% of that of a standard preparation; protein was absent from all preparations.

The results of seven similar fractionation experiments are shown in Fig 4 in graphic form. This diagram contrasts the molar sums of adenine an thymine found in each significant fraction with the corresponding sums o guanine and cytosine. It will be noticed that the gradual extraction of th nucleohistone with salt solutions of increasing strength yields a series o nucleic acid fractions with diminishing concentrations of guanine an cytosine and rising concentrations of adenine and thymine. But what shoul be emphasized is that in all fractions the equimolarity of each pair of con

TABLE XV

Fractional Dissociation of Nucleohistone Preparations; Composition of DNA Fractions

Experiment No.	1			2		
Preparation	N-NH			R-NH		
Protein/P, weight ratio	11.5			12.7		
DNA fraction No.	I	II	III	I	II	III
NaCl molarity	0.65	0.90	2.6	0.65	0.90	2.6
% of nucleohistone P	30.1	53.5	14.6	32.4	48.0	11.0
Total P, %	8.9	9.2	8.9	9.0	9.0	8.8
Extinction, ϵ(P)	6800	6700	6850	6400	6500	6650
Viscosity, $\eta_{sp.}$(P)	525	470	420	525	450	410
Moles per 100 g.-atoms P*						
Total recovery	93.0	96.0	93.3	94.1	98.9	94.1
Adenine	26.0	30.3	31.4	26.4	30.7	30.1
Guanine	23.7	19.8	18.6	23.8	19.3	19.9
Cytosine	24.7	20.9	19.7	24.5	20.4	21.2
Thymine	25.6	29.0	30.3	25.3	29.6	28.8
Molar ratios						
Adenine + thymine to guanine + cytosine	*1.07*	*1.46*	*1.61*	*1.07*	*1.52*	*1.43*
Adenine to thymine	1.02	1.04	1.04	1.04	1.04	1.04
Guanine to cytosine	0.96	0.95	0.94	0.97	0.95	0.94
Purines to pyrimidines	0.99	1.00	1.00	1.01	1.00	1.00

* The total average recovery of moles of nitrogenous constituents per 100 g.-atoms of P is given in the first line. The mean proportions of each constituent have been corrected to a 100% recovery.

stituents and of total purines and pyrimidines—a characteristic feature of all deoxypentose nucleic acids, as will be discussed later in this chapter—is fully maintained. (See also Table XV.)

In later studies these fractionation experiments were extended to many nucleic acid preparations from the tissues of ox, pig, and man, from sea urchin sperm, and from the r$^+$ strain of coliphage T6.[259] In these experiments, and in subsequent studies with pneumococcal transforming preparations, artificially prepared complexes with histone, or in a few cases with globin, served as the starting material. A selection of such fractionation experiments is shown in Fig. 5. The results are in agreement with those obtained with calf thymus nucleohistone.

Of particular interest is the distribution of the pyrimidine satellite 5-methylcytosine in the fractions thus obtained, as shown in Table XVI. It will be noticed that significant divergences in the concentration of this

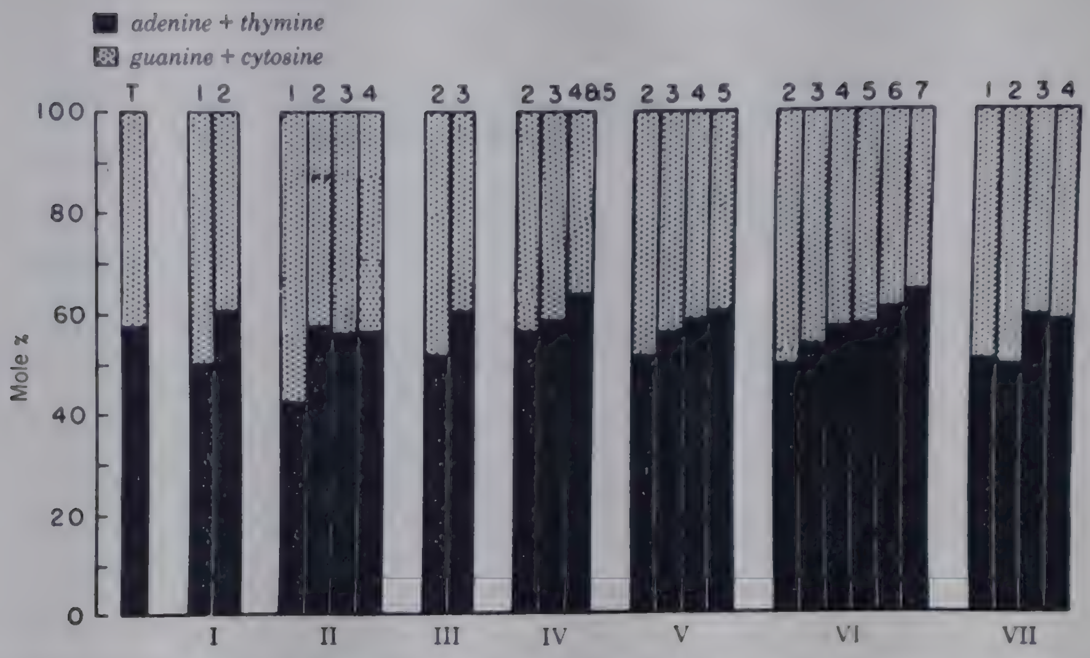

FIG. 4. Composition of sodium deoxyribonucleate fractions (in moles per 100 g. atoms of phosphorus) prepared by the fractional dissociation of calf thymus nucleohistone with salt solutions of increasing strength. Each block (I–VII) represent a separate fractionation run; the consecutively numbered bars within each bloc represent individual fractions, with the NaCl concentration rising from left to right The first column (T) indicates the average composition of the total deoxyribonuclei acid. (Taken from Chargaff *et al.*[258])

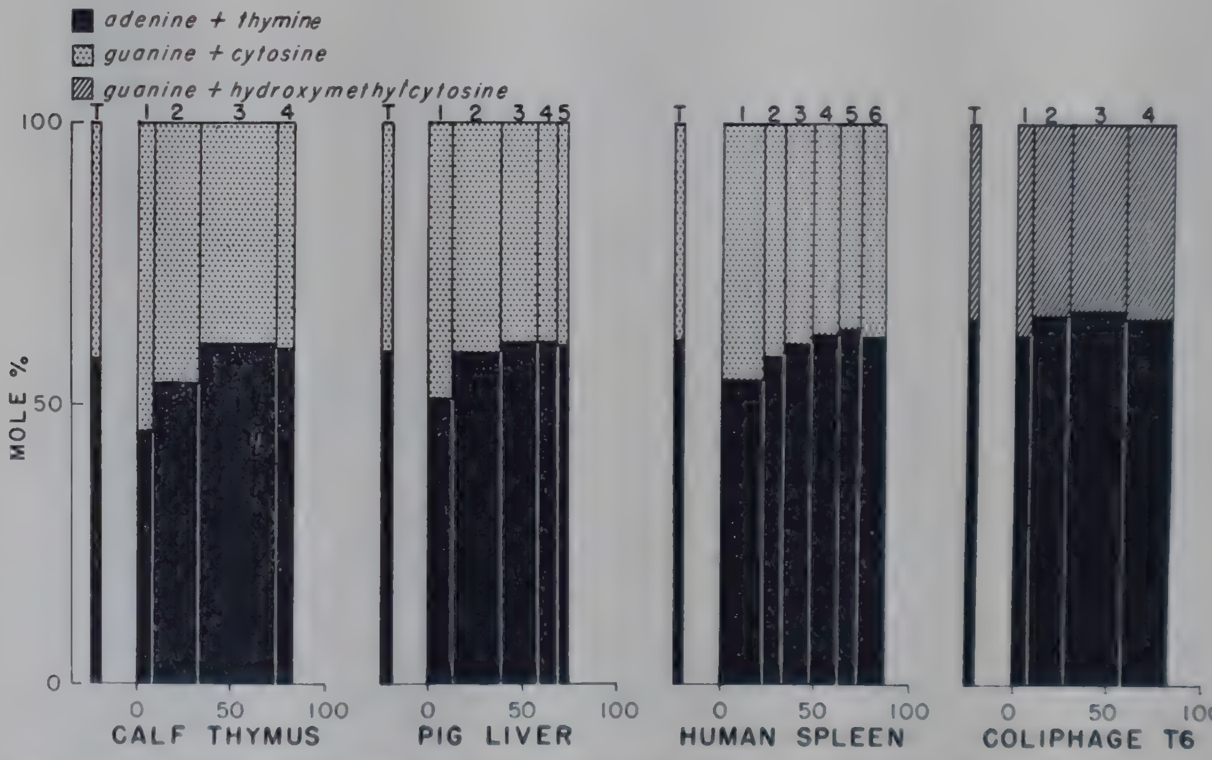

FIG. 5. Composition of fractions (in moles per 100 g.-atoms of phosphorus) prepared by the fractional dissociation of artificial histone complexes with the indicated sodium deoxypentose nucleate preparations. The abscissa indicates the proportion of original nucleic acid P recovered in each fraction. Compare Fig. 4 for other explanations. (Based on the results of Crampton *et al.*[259])

TABLE XVI

5-METHYLCYTOSINE DISTRIBUTION IN FRACTIONS OF CALF THYMUS SODIUM DEOXYRIBONUCLEATE*

	Fraction			
	1	2	3	4
NaCl molarity in extraction of nucleohistone gel	0.40	0.45	1.7	2.6
Corrected mean proportions				
Thymine	24.2	24.0	29.0	28.7
Cytosine	23.9	23.4	19.8	21.3
5-Methylcytosine	1.9	2.7	1.2	0
Molar ratio				
Thymine to 5-methylcytosine	12.7	8.9	24.2	
Cytosine to 5-methylcytosine	12.6	8.7	16.5	

* The mean proportions of each constituent have been corrected on the assumption that one-half of the nucleic acid P is contributed by the pyrimidine nucleotide. In one hydrolysis experiment with Fraction 4 a minute amount of 5-methylcytosine (about 0.6 mole) was found.

pyrimidine in the several fractions were found. This finding, it may be pointed out, is difficult to reconcile with the structural hypothesis of Watson and Crick[262] (compare Chapter 13) which would have led to the expectation of a random replacement of cytosine by the methyl derivative. (See also Section X.2.)

It is too early to attempt a detailed consideration of the reasons underlying the fractionation procedure. That nucleic acid chains relatively rich in guanylic and cytidylic acids are detached more easily than those in which adenylic and thymidylic acids predominate indicates that some property inherent in their composition or sequence reduces the strength with which they are bound to the histone. Brown and Watson[261] have, on the basis of Cavalieri's work,[191] suggested that hydrogen-bonding between the 2-amino group of guanine and a phosphate group of the sugar-phosphate backbone of the nucleic acid could reduce the acidic properties of structures in which this type of interaction occurs frequently and thereby weaken the link between protein and nucleic acid.

The fractionation experiments, which are discussed in detail in the publications cited before,[258-261] suggest that the deoxypentose nucleic acid of a given cell is composed of a very large number of differently constituted individuals and that it is possible to achieve the resolution of this entire spectrum of structural gradations (descending contents of guanine and cytosine, ascending contents of adenine and thymine) into several distin "bands," in which all the regularities characteristic of the entire nucle acid are maintained.

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[262] J. D. Watson and F. H. C. Crick, *Nature* **171**, 737, 964 (1953); *Cold Spring Harbor Symposia Quant. Biol.* **18**, 123 (1953).

IX. Composition Studies and Structural Investigations

The problem of nucleotide sequence has frequently been mentioned i the preceding pages. (See, in particular, Section V.1.) It is the most elusiv and vexing, and also the most important, part of what may be considere as the structural investigation of the deoxypentose nucleic acids. There wa never much doubt that the principal connecting links between the mononucleotides were 3′,5′-phosphate bridges (see Chapter 12), though th proverbial exceptions that should prove or test this rule have, perhaps, no yet been looked for in a sufficient number of cases. But even complet certainty regarding the points of attachment of the phosphate bridge would contribute no more to the problem of structure than the statemen that all proteins contain peptide bonds. It must be admitted that the tas of sequence analysis, beyond our present means if a single macromolecula polynucleotide chain is to be unriddled, becomes so gigantic, if the conclusions from the fractionation experiments (Section VIII) are justified as to discourage the most sanguine of optimists.

At the present time, only the crudest form of mapping the order in whic the mononucleotides are aligned in a nucleic acid chain appears attainable The task is similar to that of an ancient geographer: no more than din contours, vague directions can be discerned.

As has been pointed out before (Section V), several partial degradatio products of deoxypentose nucleic acids appear to offer an opportunity o searching for the existence of certain general structural features; these ar the apurinic acids,[170] which can be prepared readily from many deoxypentose nucleic acids, and the various large fragments formed by enzymi attack.[212, 227] If the production of pyrimidine deoxyribonucleoside diphosphates[218, 263-266] (see also Chapters 4 and 12) by acid hydrolysis of nuclei acids can be standardized and a procedure for the quantitative estimatio of individual diphosphates developed, this may also contribute to the characterization of structural differences, provided the method is applied t several purified deoxypentose nucleic acids of different origin rather tha to commercial material. If these diphosphates really are indicative of thos positions in the original nucleic acid chain in which a pyrimidine nucleotid is flanked by purine nucleotides,[266] it is conceivable that different nuclei

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[263] P. A. Levene and W. A. Jacobs, *J. Biol. Chem.* **12,** 411 (1912).

[264] P. A. Levene, *J. Biol. Chem.* **48,** 119 (1921); **126,** 63 (1938).

[265] S. J. Thannhauser and G. Blanco, *Z. physiol. Chem.* **161,** 116 (1926).

[266] C. A. Dekker, A. M. Michelson, and A. R. Todd, *J. Chem. Soc.* **1953,** 947.

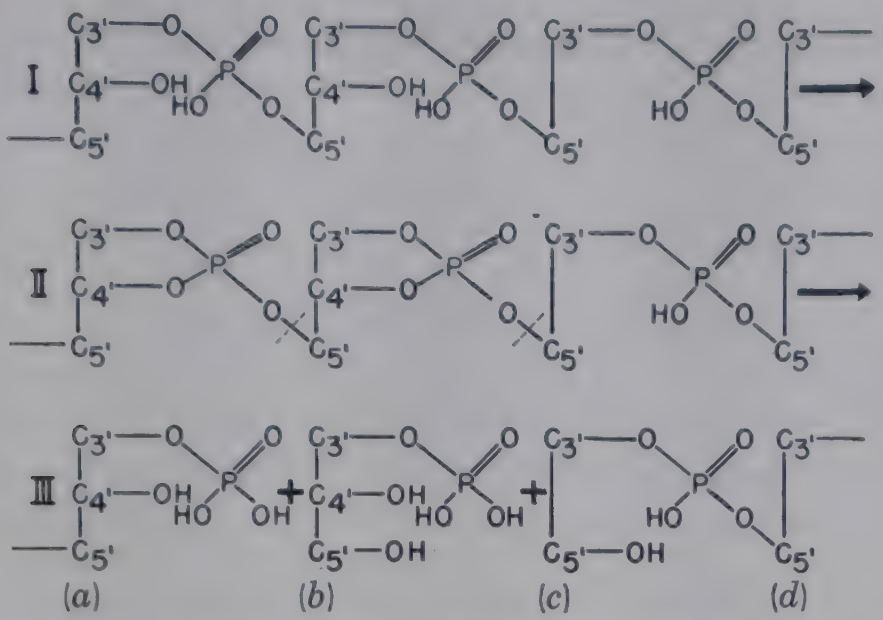

FIG. 6. Cleavage of apurinic acid by alkali. (Taken from Tamm *et al.*[221])

acids will yield entirely different results according to the particular nucleotide arrangement.

Up to this time, only the apurinic acid of calf thymus has been investigated in some detail. While enzymic investigation methods were of little avail—deoxyribonuclease apparently does not attack a chain that has been deprived of its purines[183, 225]—further degradation by alkali yielded some results.[221] Following a mild treatment with alkali the apurinic acid is cleaved in part with the formation of diffusible fragments and of a nondiffusible residue. The nondialyzable fraction comprises about 85% of the pyrimidine nucleotides, but only about 40% of the nonglycosidic deoxyribophosphate residues present in the starting material. If the sodium salt of apurinic acid is assigned an average molecular weight of 15,000,[224] a "statistical polynucleotide" in accord with its composition and properties would comprise 17 thymidylic acid residues, 12 cytidylic acid residues, and 29 deoxyribophosphate residues. About 10^{24} isomers are possible. As a plausible explanation of the partial degradation of apurinic acid by alkali a mechanism similar to that proposed for the cleavage of ribonucleic acid[267] (compare, however, Lipkin *et al.*[268]) was contemplated in which the formation, and subsequent rupture, of a transitory cyclic triester of phosphoric acid linking the 3′- and 4′-hydroxyls of the sugar phosphate residues occurring in the aldehydo form was assumed (Fig. 6). A different explanation of this reaction is given in Chapter 12. The experiments were interpreted as permitting the formulation of calf thymus deoxyribonucleic acid as

$$[(Pu_{17}Thy_2Cy_2)\ (Thy_{15}Cy_{10}Pu_{12})]_n\ ,$$

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[267] D. M. Brown and A. R. Todd, *J. Chem. Soc.* **1952,** 52.

[268] D. Lipkin, P. T. Talbert, and M. Cohn, *J. Am. Chem. Soc.* **76,** 2871 (1954).

with the first part of the expression representing the portion of apurin acid sensitive to alkali and Pu, Thy, Cy denoting purine nucleotides an thymidylic and cytidylic acids respectively. Whether the structure propose for calf thymus nucleic acid, namely, that of a chain in which trac consisting principally of pyrimidine nucleotides are followed by stretch in which purine nucleotides predominate, will be borne out by furthe work, remains to be seen. As regards the nondiffusible residue produce by the action of alkali on apurinic acid, it may be of interest to note tha about one-half remains nondialyzable even against 2 *M* NaCl solution an that an almost quantitative yield of the corresponding nucleosides is af forded by the action of phosphodiesterase and phosphomonoesterase.[269]

X. Correlations and Concluding Remarks

1. Simplifying Generalizations

Even that master of inductive reasoning, Macaulay's infant who "is le by induction to expect milk from his mother or nurse, and none from hi father," would have rejected as spurious some of the generalizations abou nucleic acids that have been made in the past and are still found in som textbooks. The effort to force nature into a strait-jacket of puerile approxi mations has yielded many short-term successes in the natural sciences. A is true of soap sculpture, they were pretty, but easily washed away. Whethe the attempt to teach the "gesta Dei per mathematicos" in three easy lesson was more harmful than useful, may remain a matter of controversy. No is plausibility a criterion of durability; quite the contrary: the gospels o the future often are the heresies of the present. But the writers of textbook thrive on premature explanations.

The "tetranucleotide theory" continues, for this reason, to lead a stub born existence. There never were any but psychological reasons for it formulation, as has been pointed out before;[4,114] but even in a very recen and massive treatise there will be found the statement that thymus nuclei acid is a large chain consisting of 500 to 1000 tetranucleotide units and tha each tetranucleotide is formed by the combination of four nucleotides con taining adenine, cytosine, guanine, and thymine, respectively.[270] Actually a glance at the tables in which the information on the composition of many different deoxypentose nucleic acids is assembled (Tables V–XIII) wil show that out of almost 50 different species so far investigated only *E. col* (Nos. 5–9 in Table XII) has yielded preparations that could be called "sta tistical tetranucleotides," though not much good will come of it. One mus conclude that there is no subunit of recognizably recurrent structure larger than a mononucleotide.

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[269] M. E. Hodes and E. Chargaff, in preparation.

[270] B. Flaschenträger and E. Lehnartz (eds.), "Physiologische Chemie," Vol. I, p 768. Springer, Berlin, Göttingen, Heidelberg, 1951.

2. Unifying Generalizations

The fear of premature generalizations, though justified on the whole, is not without its own risk. By insisting only on the differences between the various deoxypentose nucleic acids, of which there are many, one may not see the unity for the decimals. The deoxypentose nucleic acid molecules appear to possess no perceptible periodicity of their constituents: the nucleotide sequence is probably arrhythmic. If some recurrent features exist, we have no means of discerning them, as a bird's-eye view of this giant throw of dice requires a distance which is denied us. It is, therefore, the more surprising that the inspection of even the earliest analytical results compelled the recognition of several regularities which, since the time when they were first proposed,[4] have become well established: (a) The sum of the purine nucleotides equals that of the pyrimidine nucleotides. (b) The molar ratio of

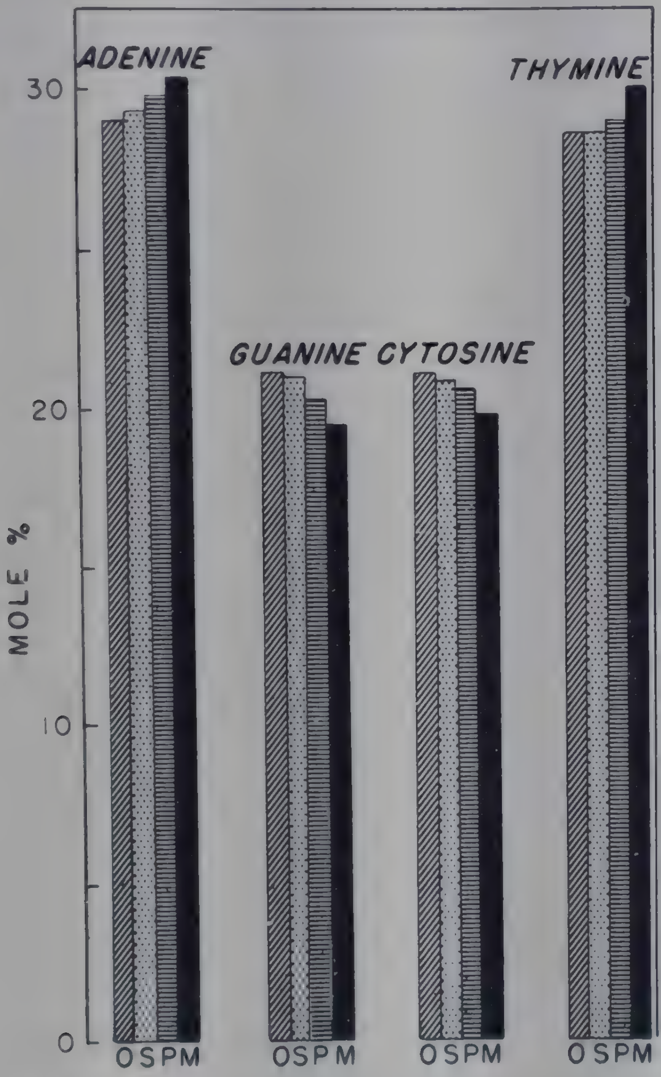

FIG. 7. Proportions of nitrogenous constituents (per 100 g.-atoms of phosphorus) in the deoxyribonucleic acids of the ox (O), sheep (S), pig (P), and man (M). (Taken from Chargaff and Lipshitz.[111])

TABLE XVII

MOLAR RELATIONSHIPS IN ALL DEOXYPENTOSE NUCLEIC ACIDS*

Table No.	Number of entries	Adenine to thymine	Guanine to cytosine	Purines to pyrimidines	6-Amino to 6-keto group
V	10	1.020±0.007	0.997±0.004	1.010±0.005	1.013±0.004
VI	4	1.015±0.003	0.995±0.006	1.010±0.004	1.013±0.003
VII	15	1.007±0.011	1.015±0.022	1.008±0.010	0.999±0.011
VIII	10	0.998±0.009	1.016±0.014	1.002±0.007	0.995±0.008
IX	14	1.034±0.013	1.014±0.012	1.023±0.008	1.014±0.009
X	17	1.006±0.012	0.980±0.020	0.995±0.013	1.013±0.010
XI	4	0.985±0.010	0.968±0.057	0.975±0.025	1.010±0.032
XII	17	1.016±0.024	0.953±0.022	0.974±0.018	1.042±0.017
XIII	10	0.974±0.007	1.083±0.007	1.006±0.008	0.955±0.004
All tables (weighted mean)	101	1.009	1.001	1.000	1.008

* The mean ratios and their standard errors are given for each table. In Table VII, Nos. 1, 2, and 11 wer not included; in VIII, No. 1; in IX, Nos. 11, 15, and 16; in XII, Nos. 1, 2, and 15. The values for cytosin refer also to 5-methylcytosine or 5-hydroxymethylcytosine.

adenine to thymine equals 1. (c) The molar ratio of guanine to cytosin equals 1. (d) The number of 6-amino groups is the same as that of 6-ket groups.[271] It is, in fact, because of these relationships that it is possible t state that, for instance, in the deoxypentose nucleic acids of wheat germ 5-methylcytosine substitues for part of the cytosine,[167] that in certain phag nucleic acids 5-hydroxymethylcytosine replaces cytosine entirely,[151] or that as has been shown recently,[238] 5-bromo- or 5-iodouracil may under circum-stances take the place of thymine. It is not unlikely that more instances o such substitution will be discovered. What appears remarkable, howeve is that up to this time they have been found only among the pyrimidine and that no purine satellite has been revealed.

Some of these relationships are, with respect to the composition of th nucleic acids of different mammalian genera (Table VI), illustrated in Fig 7. A complete survey of almost all data assembled in Tables V–XIII i provided in Table XVII. The above-mentioned ratios were calculated separately for each entry and their average values and standard errors com-puted for each tabular division. (The assistance of Dr. D. Elson in thes calculations is gratefully acknowledged.) It will be seen that the agreement is most impressive, especially in view of the diversity of sources, preparations, and procedures.

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[271] This means that the sum of guanine and thymine equals that of adenine and cytosine (+ methylcytosine). Originally, the ratio of all amino groups to enolic hydroxyls was introduced.[167] The present expression is preferable, especially in the light of recent findings on pentose nucleic acids.[272]

[272] D. Elson and E. Chargaff, *Nature* **173,** 1037 (1954).

An attempt has been made[262] to derive a molecular structure of deoxypentose nucleic acids from these regularities and from the available X-ray evidence. [Compare the discussion in Chapter 13.] This hypothesis, which postulates a helical dyad in which the two coiled strands are held together by a specific pairing of the bases showing the unity relationships mentioned above, has much to recommend itself on aesthetical grounds; it makes good use of several experimentally established facts. Whether it does more than to describe the structure of that portion of the processed preparation from which the diffraction patterns are obtained, remains, however, to be established. It is not improbable that the scheme is incomplete in some essential features, at least insofar as substitution in position 5 of the pyrimidines is concerned. If 5-methylcytosine or analogues could take the place of cytosine and vice versa without restriction, the 6-amino pyrimidines should be able to replace each other at random. This is obviously not the case, as shown by the remarkable constancy of the 5-methylcytosine content of a given species. (See, especially, Table XI.) The absence of uracil from deoxypentose nucleic acids also is not easy to understand on these grounds. Even more disturbing, perhaps, is the fact, pointed out before (Section VIII.2 and Table XVI), that 5-methylcytosine is distributed unevenly in the fractions obtained by the fractionation of calf thymus deoxyribonucleic acid. One gains the impression that—just as certain phage nucleic acids contain 5-hydroxymethylcytosine in total replacement of cytosine—there exist nucleic acid molecules as part of the preparations from calf thymus or from wheat germ in which cytosine is entirely replaced by 5-methylcytosine, whereas other fractions are, in turn, completely devoid of the latter.

3. A CONCLUDING REMARK

It may be considered as intellectually quite unsatisfactory that a considerable part of what is known about the composition and structure of nucleic acids must, as has been shown here, rest on assiduous analytical work. In our time, much stress is laid on the *forces* that govern the life and the economy of the cell. The discovery that the cell runs while it rests has effaced the other half of the truth: that it rests while it runs. The quiet center is falling into oblivion; and there is widespread contempt for what is regarded as morphology or analysis. We should, however, comprehend that it is by way of decimals that we penetrate into nature. We read in The Wisdom of Solomon (11:21): "But thou hast ordered all things in measure and number and weight." We can only hope that the span will not be too wide, the count too high, the weight too heavy.

CHAPTER 11

Isolation and Composition of the Pentose Nucleic Acids and of the Corresponding Nucleoproteins

B. MAGASANIK

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I. Introduction

The early history of the discovery of the nucleic acids is described in Chapter 1.

The development of modern methods of cytochemistry allowed th demonstration that both types of nucleic acid are present in all cells, an mal, plant, and microbial, deoxypentose nucleic acid in the nucleus, an pentose nucleic acid in the cytoplasm as well as in the nucleus.[1] Whil early preparations of PNA usually were badly degraded, the lability c PNA was later recognized, and consequently milder methods of isolatio came into use which resulted in PNA preparations of considerably highe molecular weight.[2,3] Finally, the application of modern methods to th analysis of nucleic acids, such as paper chromatography,[4] ion-exchang chromatography,[5] and paper ionophoresis,[6] demonstrated that nuclei acid preparations isolated from different tissues differed in compositio and did not in general contain the four constituent nucleotides in equimola quantities.

The description of these newer methods of isolation and the result obtained by the modern methods of analysis are the subject of this chapter.

II. Isolation of Pentose Nucleoproteins

1. General

Pentose nucleic acids are found in tissue extracts in combination wit proteins as nucleoproteins, the "nucleins" of the early workers. Generally isolation of nucleoprotein is the first step in the isolation of PNA.

A short description of the distribution of PNA in the cell, a subjec discussed in detail in Chapters 16–21, is helpful for the understanding o the experimental approach used for the isolation of nucleoproteins. PN is found in both nucleus and cytoplasm. The nuclear PNA accounts fo about one-tenth of the total PNA of the cell.[7] The isolation of nucleoli anc the composition of their PNA constituent has been described.[8] The majo portion of the PNA of the cytoplasm is contained in the particulate fractions, and most of it is found in the microsomes. Chemical analysis ha shown microsomes to be complex macromolecular structures composed o lipid, protein, and PNA.[9] The fractionation of the components of the microsomes without the denaturation of the protein has not been accomplished. The soluble PNA found in the supernatant fluid after sedimentation of the microsomes is also bound to protein.[10]

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[1] T. Caspersson and B. Thorell, *Chromosoma* **2,** 132 (1941).

[2] H. S. Loring, *J. Biol. Chem.* **130,** 251 (1939).

[3] W. E. Fletcher, J. M. Gulland, D. O. Jordan, and H. E. Dribben, *J. Chem. Soc* **1944,** 30.

[4] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176,** 715 (1948).

[5] W. E. Cohn, *J. Am. Chem. Soc.* **72,** 1471 (1950).

[6] R. Markham and J. D. Smith, *Biochem. J.* **52,** 552 (1952).

[7] W. C. Schneider, *J. Biol. Chem.* **165,** 585 (1946).

[8] W. S. Vincent, *Proc. Natl. Acad. Sci. U. S.* **38,** 139 (1952).

[9] A. Claude, *Science* **97,** 451 (1943).

The PNA-lipoprotein complexes which constitute the particulate fractions of animal cells may be isolated by fractional centrifugation of tissue homogenates. Analogous procedures may be used for the isolation of some animal viruses and of all plant viruses which resemble the cytoplasmic particles in size, composition, and distribution in the host cell. Alternatively, the tissue may be extracted with isotonic saline and the cytoplasmic PNA-proteins isolated by isoelectric precipitation of the extracts at a pH of about 5. The soluble PNA-proteins as well as the microsomes and mitochondria are obtained together in this procedure. This method can be used for the isolation of PNA-proteins from the organs of animals and from yeast, and for the isolation of plant viruses. Other methods, such as the fractionation of tissue extracts with ammonium sulfate or sodium sulfate, have been useful in special cases.

2. Isolation of Nucleoprotein from Animal Tissues

a. *Fractional Centrifugation*

This method for the isolation of the cytoplasmic nucleoproteins is described in Chapter 21. Similar procedures were used for the isolation of the virus of equine encephalomyelitis from infected chick embryos by Taylor *et al.*[11] The virus was found to be composed of protein, phospholipid, and PNA.

b. *Extraction with 0.14 M Sodium Chloride Followed by Isoelectric Precipitation*

This method takes advantage of the fact that PNA-protein but not DNA-protein is readily soluble in 0.14 *M* sodium chloride. The PNA-protein is subsequently precipitated by addition of acid to a pH of 4–5. The preparation of calf thymus PNA-protein by this procedure has been described by Mirsky and Pollister,[12] and that of pancreas PNA-protein by Kerr and Seraidarian.[13] These preparations consisted of mixtures of particulate and soluble nucleoproteins, and were not further characterized but used directly for the preparation of free PNA.

c. *Salt Fractionation*

This method has not been used extensively. It does, however, deserve attention because of its recent successful application by Hamoir to the isolation of a crystalline PNA-protein from carp muscle.[14] This compoun nucleotropomyosin, is the first crystalline nucleoprotein to be prepare from animal tissue and possesses considerable chemical and biologic interest. Together with nucleotropomyosin, another crystalline protein, th well-known muscle component tropomyosin, was obtained. The two proteins were indistinguishable by electrophoresis but behaved differently i the ultracentrifuge. Tropomyosin was free from PNA and identical wit the protein portion of nucleotropomyosin. In contrast to the comple cytoplasmic nucleoproteins isolated so far, nucleotropomyosin appears t be free of phospholipids. The method of preparation is given in detail.

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[10] D. Szafarz, *Biochim. et Biophys. Acta* **6,** 562 (1951).

[11] A. R. Taylor, D. G. Sharp, D. Beard, and J. W. Beard, *J. Infectious Diseases* **72,** 31 (1943).

[12] A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.* **30,** 101 (1946).

[13] S. E. Kerr and K. Seraidarian, *J. Biol. Chem.* **180,** 1203 (1949).

(1) *Nucleotropomyosin from Carp Muscle.*[14] The preparation is carried out throug out in the cold and all separations are done by centrifugation. Carp muscles cu with a freezing microtome into slices 40 μ thick are extracted for 20 minutes wit 3 vol. of KCl–phosphate solution (0.50 *M* KCl and 0.1 *M* KH_2PO_4 brought to pH 5.) This extract is diluted with 3.5 vol. of cold water; the precipitate is discarded and th supernatant (I) kept.

The residue from the first extraction is reextracted for 10 minutes with 3 vol. o 0.5 *M* phosphate solution of pH 5.5 containing 0.3% sodium adenosinetriphosphate The residue is discarded and the extract (II) is diluted with 7 vol. of water. A precipitate of myosin and nucleotropomyosin forms, which is washed twice with wate and redissolved in 0.5 *M* KCl at neutral pH. The supernatant II and the supernatan I are mixed and brought to 4.6. The precipitate containing tropomyosin is washe twice with water and redissolved in 0.5 *M* KCl at neutral pH.

Both solutions are now centrifuged for 30 minutes at 14,000 r.p.m. to remove som turbid material and are purified by a second precipitation by dilution with 8 vol. o water at neutral pH (nucleotropomyosin) or at pH 4.6 (tropomyosin). Both precipitates are washed twice with water and redissolved in 0.5 *M* KCl at neutral pH.

Both tropomyosins are isolated from these two solutions by $(NH_4)_2SO_4$ fractionation at neutral pH: the major part of the total protein content of the solutions precipitates between 30 and 50% saturation, while the tropomyosins precipitate betwee 50 and 66% saturation. The precipitate can be redissolved quickly by a slight dilution with water, giving a water-clear solution.

Although these methods of preparation are very reproducible, some variations ar observed in the yields obtained, which are usually 0.07% of the wet weight for nucleotropomyosin and 0.03% for tropomyosin.

The undiluted salted-out precipitates are used for crystallization. This is carrie out by dialyzing an approximate 1.5% solution against a solution containing 16 g $(NH_4)_2SO_4$ per liter and 0.01 *M* acetate buffer of pH 5.4. Nucleotropomyosin crystallizes in elongated prisms; tropomyosin in the quadrangular plates previously described by Bailey.[15]

3. ISOLATION OF NUCLEOPROTEIN FROM THE TISSUES OF HIGHER PLANTS

a. Fractional Centrifugation

This method has been used extensively for the isolation of plant viruses from leaves. The mildness of the procedure permits the isolation of some of the less-stable viruses which do not survive chemical fractionation procedures. The success of this method for the purification of plant viruses depends on the absence of material of corresponding particle size and stability in uninfected plants. A nucleoprotein of the size of plant viruses has been isolated by Pirie[16] from the sap of young uninfected tobacco leaves. However, the amount of this material in older leaves is very small, and it is removed in the course of the procedures used to clarify the infected sap prior to the sedimentation of the plant virus. Consequently, plant viruses isolated from cell sap, clarified by precipitation with ethanol, freezing, and exposure to inorganic phosphate, will not be contaminated by the nucleoprotein of normal plant leaves.[16]

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[14] G. Hamoir, *Biochem. J.* **48,** 146 (1951).

[15] K. Bailey, *Biochem. J.* **43,** 271 (1948).

Several strains of tobacco mosaic and related viruses have been isolated by fractional centrifugation for a study of the amino acid and nucleotide composition of the virus nucleoprotein.[17-19] The preparation of cucumber virus 4 (CV4) is given in detail.

(1) *Preparation of Cucumber Virus 4 (CV4)*[17]: Young cucumber plants were inoculated with CV4 by rubbing one leaf on each plant with a gauze pad saturated with infective juice. This juice was obtained from a cucumber plant showing the typical yellow mottling produced by cucumber virus 4 in members of the Cucurbitaceae. About 1 month after inoculation, the plants were harvested and placed in a room kept at −12°. After a few days the frozen plants were ground. Three per cent by weight of dipotassium phosphate in a 50% solution was mixed with the pulp, and after about 2 hours the juice was expressed from the cold but completely thawed pulp. The juice was passed through a celite filter to remove coarse particles of green pigment and extraneous matter, and then the virus was sedimented in the form of pellets by centrifugation at 20,000 to 30,000 r.p.m. for 30 minutes. The supernatant liquid, which was practically free of virus, was discarded, and the pellets were dissolved in small amounts of distilled water. The combined solutions of virus pellets were spun at about 3000 r.p.m. on an angle centrifuge for 30 minutes to remove green pigment and insoluble colloidal matter. The supernatant liquid, which contained the virus, was then returned to the high-speed centrifuge and the process was repeated about three times, a longer period being allowed for the high-speed centrifugation as the virus became more concentrated. In many cases it was found possible to effect a better separation of green pigment from the virus by dissolving the pellets obtained by the first two high-speed centrifugations in 0.1 M phosphate buffer at pH 7 and then using distilled water as a solvent for the pellets of the last two centrifugations. All of the preparations used for elementary analysis were further purified by dialysis against flowing distilled water for 48 hours.

Pellets of purified virus ranged from 0.1 to 0.4 g. per liter of expressed juice as compared with 2–2.5 g. per liter ordinarily obtained for tobacco mosaic virus from the juice of diseased turkish tobacco plants.

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[16] N. W. Pirie, *Biochem. J.* **47,** 614 (1950).

[17] C. A. Knight and W. M. Stanley, *J. Biol. Chem.* **141,** 29 (1941).

[18] C. A. Knight, *J. Biol. Chem.* **171,** 297 (1947).

[19] C. A. Knight, *J. Biol. Chem.* **197,** 241 (1952).

b. Salt Fractionation

The original isolation of tobacco mosaic virus was achieved by salt fractionation.[20] The procedure recently described for the preparation of turnip yellow mosaic virus by fractional precipitation with ammonium sulfate is given in detail.[21]

(1) *Preparation of Turnip Yellow Mosaic Virus.*[21] The virus has been prepared from a number of cruciferous plants, namely radish, *Bronica carrinata*, turnip, chinese cabbage, and from a specimen of broccoli found infected naturally. For general use either turnip or chinese cabbage is suitable and, generally speaking, old or "hard" plants give better yields than rapidly growing "soft" plants. Yields up to 1 g. of virus per liter of sap have been obtained from stunted turnip plants grown in the open, but the usual yields from glasshouse plants vary from 200 to 500 mg. per liter.

Plants infected with the virus are minced, either fresh or after freezing overnight, and the sap is expressed. If field turnips are used, it is necessary to use a hydraulic press to remove the last half of the sap. The pH of the sap is usually about 5.7. The sap is clarified by adding slowly, with stirring, 300 cc. of 90% ethanol to each liter. This quantity of ethanol is fairly critical and should be measured carefully.

The copious precipitate which forms is centrifuged off at once at 3500 r.p.m. for 15 minutes. To the supernatant liquid, a volume of saturated ammonium sulfate equal to half the volume of purified sap is added, and the solution is allowed to stand, preferably overnight. After a few hours, large numbers of small octahedral crystals are to be found in the liquid, and these increase in size with time. Many small, highly refractive, birefringent crystals are also to be seen, especially in the sap from old turnip plants, but these are inorganic and are removed later.

The crystalline precipitate is centrifuged off (30 minutes at 3500 r.p.m.), but the supernatant liquid frequently deposits a second crop of crystals on standing for a day or two, and so may be kept. In addition, it is very difficult to remove all the small crystals by centrifuging as they sediment rather slowly because they are not very dense.

The precipitate which contains the virus and much insoluble material is resuspended in a small volume of water (one-tenth to one-quarter of the original sap volume) or buffer solution, and is centrifuged again to remove insoluble material. Precipitation of the virus followed by resolution of the crystals and centrifuging to remove debris is repeated several times. In favorable circumstances the virus preparation may be fairly clean by this stage.

Several methods may be used for further purification. The virus is not digested by trypsin and treatment with commercial pancreatic extract (10 mg./cc.), and incubation for a few hours with a few drops of chloroform as preservative will remove some of the contaminants. The enzyme is removed by several recrystallizations of the virus.

A treatment which is of use in removing brown pigment, if present, is crystallization of the virus from dilute alcohol solutions. A solution containing some 5 mg./cc. of virus and a trace of salt is cooled to 0° C. and 0.25–0.30 vol. of absolute alcohol is added gradually with stirring. On adding drop by drop a solution containing 20 cc. of absolute alcohol, 10 cc. of glacial acetic acid, and water to make 100 cc., the virus solution becomes turbid, and this turbidity disappears on allowing the solution to warm. If the solution is left in the refrigerator for a few hours, the virus crystallizes out in the form of small birefringent needles; they may be left at 1° C. for a few days, when the mass of crystals will be found to adhere to the bottom of the container and the mother liquor may be decanted without difficulty. The crystals are then dissolved in 0.1 M disodium phosphate or a neutral buffer, as they form cloudy suspensions in plain water. Spinning at 5000 r.p.m. removes the pigmented material which by now is insoluble.

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[20] W. M. Stanley, *Science* **81,** 644 (1935).

[21] R. Markham and K. M. Smith, *Parasitology* **39,** 330 (1949).

4. Isolation of Nucleoprotein from Microbial Tissues

The protoplasm of microorganisms is especially rich in nucleoprotein. Bacteria contain as much as 15% (dry weight) nucleic acid, of which 60–75% is of the pentose type. In spite of the high concentration of PNA in bacterial cells, it is difficult to obtain bacterial PNA-protein preparations free from DNA-protein and comparable in purity to the PNA-proteins isolated from animal cells. The obstacles encountered are the difficulty of breaking up bacterial cells without harm to their labile constituents, the presence of potent nucleases in bacterial extracts, and finally, the presence of DNA-protein throughout the cytoplasm rather than in a discrete removable structure such as the nucleus.

Recently, Parsons has described the isolation of a PNA-protein free from deoxyribose from *Clostridium perfringens*.[22] The nucleic acid was extracted from lyophilized cells with 0.14 M NaCl and purified by precipitation with methanol at −10° C. and with ammonium sulfate.

The situation is more favorable in yeasts primarily because of their low DNA-protein content. Thus the isolation of PNA-proteins from acetone-dried ground yeast has been accomplished by Khouvine.[23] Several nucleoproteins which differed in nucleotide composition were obtained by isoelectric precipitation at different pH's. The significance of this observation will be discussed in the section on nucleotide composition.

III. The Nature of Pentose Nucleoprotein

The isolation of material consisting of nucleic acid and protein is in itself no proof that an entity such as "nucleoprotein" really exists. The material isolated may be a conglomerate consisting of a random collection of distinct protein and PNA units held together by the attraction of oppositely charged groups located on their surfaces. Such salt-like combinations of protein and PNA could be present in the cell, or the two components could meet and become attached to one another in the course of the isolation procedure. In either case, the proper approach would be to separate the protein and nucleic acid moieties and to study their individual properties.

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[22] C. H. Parsons, Jr., *Arch. Biochem. and Biophys.* **47,** 76 (1953).

[23] Y. Khouvine and H. De Robichon-Szulmajster, *Bull. soc. chim. biol.* **33,** 1508 (1951).

On the other hand, nucleoproteins may be well-defined chemical structures in which nucleic acid and protein are arranged in a definite pattern and these entities may be the ultimate carriers of biological properties The detachment of the PNA from the protein would then present us with fragments valuable for chemical and physical study but without biologica interest.

The question of the nature of PNA-protein is thus largely a question o the nature of the bonds which hold PNA and protein together. Certain relevant conclusions may be drawn by examining the conditions which cause these bonds to break.

The early nucleic acid chemists used dilute alkali to separate PNA from protein, but with the recognition of PNA as an alkali-labile polymer, other methods came into use. The most generally applicable of these methods is treatment with hot 10% sodium chloride.[24] Recently milder procedures, such as treatment with guanidine hydrochloride[25] or with sodium dodecyl sulfate[26] in the cold, have been found effective in separating PNA from protein. In the special case of the plant viruses even milder treatment suffices: the PNA of tobacco mosaic virus was liberated by very short heating at neutral pH;[27] the PNA of turnip yellow mosaic virus could be detached from the protein by treatment with 30% ethanol in the cold.[21]

It is quite evident from the ease with which nucleoprotein can be split that the two moieties are not joined by covalent bonds. The agents used for the separation of PNA from protein are all effective protein denaturants, and, indeed, the removal of PNA is always accompanied by the denaturation of the protein. It seems likely, therefore, that the forces which are responsible for keeping protein molecules in their native folded configuration are also responsible for the binding of PNA to protein. These forces are the coulombic attractions of oppositely charged ions, the attraction of dipoles, and hydrogen bonds. The polar groups on protein and PNA are well suited for such mutual attractions.

These forces of attraction may be quite nonspecific and cause the formation of nucleoprotein whenever PNA and protein are in close proximity. Alternatively, nucleoproteins may be formed just like native proteins, under the influence of specific directing forces active in the cell. Recent work sheds some light on this problem, although sufficient evidence which would enable us to choose between these alternatives has not been obtained. Szafarz[10] observed that the supernatant fluid of cytoplasmic extracts centrifuged at 60,000 *g* did not contain a component corresponding

[24] J. N. Davidson and C. Waymouth, *Biochem. J.* **38,** 375 (1944).

[25] E. Volkin and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1516 (1951).

[26] E. R. M. Kay and A. L. Dounce, *J. Am. Chem. Soc.* **75,** 4041 (1953).

[27] S. S. Cohen and W. M. Stanley, *J. Biol. Chem.* **144,** 589 (1942).

in electrophoretic mobility to free PNA. The pH of the extracts could be varied from 5.7 to 8.2 and the ionic strength from 0.08 to 0.31, without resulting in the appearance of a band characteristic of free nucleic acid. Szafarz therefore concluded that the nonsedimentable fraction of cytoplasmic PNA is bound to protein by bonds stable over a considerable range of pH and ionic strength. In contrast, the bonds between simple proteins such as albumin and PNA, which are formed when the two components are mixed at a pH intermediate between their isoelectric points, are not stable in solutions of an ionic strength equivalent to that used in the experiments with the cytoplasmic nucleoproteins.[28] The bond in the cytoplasmic nucleoprotein may owe its strength to the particular properties of either the protein or the PNA, or to a particular steric relation between the two components. An interesting observation by Szafarz[10] suggests that proteins of the cytoplasm possess special ability to form stable complexes with PNA. Thus, small amounts of commercial yeast PNA added to cytoplasmic extracts of the flagellate *Polytomella coeca* could not be demonstrated by electrophoresis of the mixture over an extended range of pH and ionic strength. The proteins of the cytoplasm were capable of reacting *in vitro* with PNA isolated from a different species, and the resulting complex possessed the stability of a cytoplasmic nucleoprotein. The study of such artificial nucleoproteins may lead to the discovery of the structural properties of cytoplasmic proteins which enable them to combine with PNA. At present, it would seem most likely that the charged groups on the surface of protein and PNA molecules are responsible for the ease of the interaction, and that the great number of such coulombic bonds between each molecule of protein and PNA accounts for the stability of the nucleoprotein complexes.

It is evident that free PNA could not be found in tissue extracts in the presence of reactive cytoplasmic protein, even if it did exist in that state in the intact cell. Without further evidence, the nucleoproteins isolated from cytoplasmic extracts cannot be considered to be characteristic components of the intact cell.

However, nucleoproteins which differ in properties from the complexes formed by the direct interaction of protein and PNA seem to exist. An interesting example is the crystalline nucleotropomyosin isolated by Hamoir from carp muscle[14] by the method described in detail in the preceding section. The two crystalline proteins, tropomyosin and nucleotropomyosin, showed identical behavior in an electric field, although the former was free of nucleic acid while the latter contained 15% PNA. The presence of nucleic acid thus did not affect the charges on the surface of the protein, and it must be assumed that the PNA fits into the pattern of the protein in such a manner that its charged groups are not exposed an the charged groups on the surface of the protein are not affected. Such a orderly arrangement of protein and PNA would not be expected to resul from the random interaction of the polymers without the influence of directing force. Nucleotropomyosin does not have a fixed composition.[2 Exposure of this nucleoprotein to low pH results in the liberation of tropomyosin and in the accumulation of nucleotropomyosin of increased nuclei acid content. Within large limits (10–20% of PNA) the physical propertie of these compounds which depend on surface charges are identical. When the PNA content is increased to 30%, a nucleoprotein of lower isoelectri point than the original nucleotropomyosin precipitates.

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[28] L. G. Longsworth and D. A. MacInnes, *J. Gen. Physiol.* **25**, 507 (1942).

The properties of nucleotropomyosin are those of a compound in which a core of PNA is completely covered by protein. Apparently a large portion of the protein can be peeled off without exposing the charged groups of the PNA core, and therefore without altering those physical properties of the complex which depend on surface charges.

A similar relationship between protein and PNA has been discovered in one of the crystalline plant viruses, turnip yellow mosaic.[21] The sap of infected plant leaves contains, in addition to the virus nucleoprotein, another protein free of PNA but of similar size, shape, and crystal structure, and with identical electrophoretic behavior. The two proteins could be separated by ultracentrifugation. They reacted quantitatively in the same manner with antiserum directed against the nucleoprotein. However, only the nucleoprotein could infect plants or cause the formation of antibodies when injected into animals. Pancreatic ribonuclease did not attack the intact nucleoprotein, but hydrolyzed the nucleic acid prepared from it in the usual fashion.

Here again it appears that the PNA contained in the nucleoprotein is completely covered by protein so that the surface properties of the complex, such as electrophoretic mobility and reaction with antibodies, are exclusively determined by the nature of the protein component. The stability of the nucleoprotein to the action of pancreatic ribonuclease confirms the assumption that the PNA moiety is not easily accessible. The biological properties of the nucleoprotein, such as infectivity and antigenicity, depend on the presence of the PNA. However the PNA alone, separated from the nucleoprotein by denaturation of the protein with dilute ethanol, is neither infective nor antigenic. It would seem that the nucleoprotein possesses activities which are not the sum of the activities of its constituent parts; in this sense the nucleoprotein is an ultimate biological unit. Still, it must be borne in mind that we do not know whether the isolated PNA is in its native state; treatment sufficiently drastic to

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[29] G. Hamoir, *Biochem. J.* **50**, 140 (1952).

TABLE I

PROPERTIES OF CRYSTALLINE PLANT VIRUS NUCLEOPROTEINS

Virus	Molecular weight × 10^{-6}	PNA, %	Nucleotides per mole	Ref.
Tobacco mosaic (TMV)	40	6	8000	30
Rib grass	40	6	8000	31
Cucumber (CV4)	40	6	8000	17
Tomato bushy stunt	10.6	17	5000	32,33
Southern bean mosaic	6.6	21	4000	34
Tobacco ring spot	3.4	40	4000	35

denature protein might well have a similar destructive effect on the integrity of PNA. The possibility cannot be ignored that native PNA, if we could obtain it, would be found to possess alone the biological activities associated with the nucleoprotein.

The composition of the *individual* plant virus nucleoprotein is constant. The material isolated from plants infected with turnip yellow mosaic virus consists of only two components: the free protein, and the nucleoprotein containing 28% PNA. Complexes of intermediate PNA content are not found. The compositions of several crystalline plant viruses are presented in Table I. The PNA content of these preparations is constant, regardless of the host, the time of harvest, or the method of isolation. It is further of interest that the different strains of tobacco mosaic virus all have identical PNA content, and even, as will be discussed later, identical nucleotide composition; however, the amino acid composition of their proteins differs.[36, 18] The particle weight of *different* plant viruses and their PNA content show great variation. Thus, tobacco mosaic virus has a particle weight of 40 × 10^6 with a PNA content of 6%, while tobacco ring spot virus with a particle weight of only 3.4 × 10^6 contains 40% PNA. If their PNA content is expressed as the number of nucleotides per virus particle, considerably less variation—only from 8000 to 4000—is observed. When the assumption is made that each virus particle contains 1 molecule of PNA, the molecular weight of tobacco mosaic PNA would be of the order of 2,500,000; actually, the molecular weight of tobacco mosaic PNA isolated by the mildest procedure[27] was estimated as 300,000. It appears

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[30] W. M. Stanley and H. S. Loring, *Relazioni del IV Congresso Internazionale di patologia comparata* **1,** 45 (1939).

[31] C. A. Knight, *J. Biol. Chem.* **45,** 11 (1942).

[32] W. M. Stanley, *J. Biol. Chem.* **135,** 437 (1940).

[33] H. Neurath and G. R. Cooper, *J. Biol. Chem.* **135,** 455 (1940).

[34] G. L. Miller and W. C. Price, *Arch. Biochem.* **10,** 467 (1946).

[35] W. M. Stanley, *J. Biol. Chem.* **129,** 405 (1939).

[36] C. A. Knight and W. M. Stanley, *J. Biol. Chem.* **141,** 39 (1941).

that either extensive depolymerization occurred when the nucleoprotei was split, or that each particle of nucleoprotein contains as many as 10 molecules of PNA.

IV. The Isolation of Pentose Nucleic Acids

1. GENERAL

It is the aim of biochemical research to relate the chemical properties of natural substances to their function in the living organism. An important early step in work of this kind is, therefore, the isolation of pure compounds which are characterized by possessing specific biological activity. In most cases the activity is first observed in the intact organism. An organ or group of cells rich in this activity is chosen as starting material for the isolation of the compound responsible for the activity. The components of the cells are fractionated by physical and chemical methods, using the quantitative measurement of the biological activity as a guide. The mildness or harshness of the isolation procedure depends on the nature of the carrier of the activity; the disappearance of the activity at any step gives immediate notice that this step must be avoided. Finally, the molecular homogeneity of the material thought to be the smallest carrier of the biological activity is investigated by physical methods.

The success of this approach in the isolation of pure proteins is, in large measure, due to the striking and easily measurable biological activity so many proteins such as enzymes, antibodies, and hormones possess. The lessons learned concerning the conditions under which their biological properties are lost, such as the action of heat, strong acids and bases, etc., could be applied to proteins with less striking biological activities. The biological importance of proteins encouraged the development of physical methods such as ultracentrifugation, electrophoresis, and the measurement of diffusion by which their homogeneity could be investigated.

The situation is quite different with regard to nucleic acids, and particularly pentose nucleic acids. At present, in spite of much work and greatly more speculation, the function of PNA is not known and its biological activity can, therefore, not be measured. One must be content to aim at the recovery of material characterized in the cell only by the chemical properties of its constituents, the purine and pyrimidine bases, pentose, and phosphate; all that can be achieved is the separation of this material from all compounds of different chemical composition. It is not known whether a particular manipulation used in the course of the isolation procedure will cause a change in the properties of the PNA which are responsible for its function in the cell. Furthermore, the homogeneity of the isolated material cannot as yet be deduced with any degree of certainty from physical measurements. The difficulty of relating the results of diffusion and sedimentation measurements on highly charged particles to their size and shape is discussed in Chapter 13. In many instances physical measurements clearly indicate the inhomogeneity of PNA preparations. So far no method has been developed which permits the fractionation of the molecular species contained in such heterogeneous preparations.

The methods used for the isolation of PNA aim at the complete recovery of the PNA contained in the biological specimen under investigation. This is usually an organ, such as liver or pancreas, a homogeneous collection of cells, such as yeast paste, or a cell fraction, such as mitochondria or microsomes. The composition and properties of the isolated preparation will be a cross-section of the properties of the various pentose nucleic acids present in this material. A comparison of the nature of the PNA obtained from different biological specimens will reflect the differences in the types of PNA predominating in each specimen. It is important to keep in mind that most methods of isolation do not, however, result in the complete recovery of the PNA contained in the material. Fractionation may thus take place in the course of isolation, and PNA samples isolated from the same starting material by different isolation procedures may actually differ in composition and properties.

As mentioned before, it cannot be decided whether a given preparation of PNA has been degraded in the course of isolation as long as the biological function of PNA is not known. Therefore, only the study of the action of the chemical and physical agents employed in the course of the isolation procedures on purified samples of PNA is helpful in choosing the least destructive path. Obviously, any treatment that causes degradation of purified PNA samples must be avoided in their preparation. In earlier work PNA was extracted from tissues by dilute alkali; however, the extreme ease with which PNA is hydrolyzed by alkali to a mixture of nucleotides shows clearly that alkali should not be used in the preparation of PNA. Dilute acid hydrolyzes PNA in a similar manner; in addition, the purine–pentose bond is labile to acid and, although heat is usually necessary to accomplish these transformations, prolonged contact with acid even in the cold should be avoided. The most troublesome factor in the isolation procedure is the presence of highly active and sturdy nucleolytic enzymes in most tissues. The best known of these enzymes is pancreatic ribonuclease;[37] it was found to accompany PNA through most of the stages of isolation and was clearly responsible for the degraded state of the PNA isolated.[38] This enzyme is still active at low temperatures and is not easily inactivated by heat. The activity of the enzyme may be competitively inhibited by heparin,[39, 40] but so far this polysaccharide has not been used to protect PNA during isolation. It is claimed that the detergent sodium dodecyl sulfate[26] or guanidine hydrochloride[25] will denature the enzyme and that the use of these substances for the separation of the PNA from protein will protect PNA from ribonuclease. The only nucleic acid specimens whose degradation by ribonuclease during isolation can be excluded with certainty are those derived from plant viruses, as the nucleoprotein which is the form in which the PNA is separated from host components is resistant to the action of nucleolytic enzymes.[21]

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[37] M. Kunitz, *J. Gen. Physiol.* **24,** 15 (1940).

[38] J. E. Bacher and F. W. Allen, *J. Biol. Chem.* **183,** 641 (1950).

The effect of the damaging agents referred to so far, viz., alkali, acid and ribonuclease, is manifested by a change in the nucleotide composition of the PNA. Other agents seem to affect only the physical properties of PNA. The PNA obtained from plant viruses, whose protein moiety was denatured by short heating or by dilute ethanol, are viscous and possess a molecular weight of about 300,000.[27] On standing, such PNA preparations break down spontaneously into smaller particles. The isolation of PNA preparations of similar viscosity, particle size, and instability from the liver of rat, rabbit, and calf, has recently been reported.[41] By analogy with the observations on DNA where viscosity and high particle weight are correlated with biological activity, it is thought that such PNA preparations are more nearly representative of native PNA than others of small particle weight.[42] The preparations of PNA of high particle weight lose their viscosity and rapid rate of sedimentation in solutions of high sodium chloride content or upon heating.[41] The use of strong electrolyte solutions and heat in the process of isolation should probably be avoided.

In the succeeding sections the newer methods used for the isolation of PNA are listed; several characteristic methods, which have served for the preparation of the nucleic acids whose composition is discussed subsequently, are presented in detail. This chapter does not intend to give an historical survey of the methods used for the isolation of PNA and will, therefore, not describe the older methods in which the PNA was extracted from tissue with dilute alkali. A description of these methods, which are still used for the production of commercial yeast PNA, is found in Levene's monograph.[43]

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[39] J. S. Roth, *Arch. Biochem. and Biophys.* **44,** 265 (1953).

[40] N. Zöllner and J. Fellig, *Am. J. Physiol.* **173,** 223 (1953).

[41] E. L. Grinnan and W. A. Mosher, *J. Biol. Chem.* **191,** 719 (1951).

[42] It should be emphasized that this is only an assumption; it may be that the viscous preparations are artifacts resulting from the aggregation of PNA molecules in the course of isolation.

[43] P. A. Levene and L. W. Bass, "Nucleic Acids." Chemical Catalog Company, New York, 1931.

2. Isolation of PNA from Animal Tissues

Chargaff *et al.* isolated PNA from the livers of several species of animals.[44] Their procedure was based on a modification of the method of Davidson and Waymouth.[24] PNA was dissociated from protein by boiling in 10% NaCl. An analogous procedure used by the same authors for the isolation of PNA from baker's yeast, is described in detail below. Similar methods were used by Tsuboi and Stowell[45] and by Davidson *et al.*[46] for the isolation of PNA from liver, and by Davidson and Smellie for the isolation of PNA from cytoplasmic fractions of the liver.[47]

PNA was prepared from beef pancreas by Kerr and Seraidarian[13] by a method in which the nucleoprotein, isolated by isoelectric precipitation, was dissociated by exposure in a half-saturated solution of NaCl for a period of 36 hours or more. The danger of degradation of PNA during isolation by tissue ribonuclease is particularly great in the preparation of PNA from pancreas. Bacher and Allen described the preparation of PNA from pancreas after removal of ribonuclease by extraction with dilute acid and acetone.[38]

A general method for the preparation of PNA from different animal tissues, including liver, spleen, thymus, and pancreas, was described by Volkin and Carter.[25] It consists of the precipitation of PNA from a cold 2 *M* guanidine hydrochloride solution in which protein remains soluble. Their procedure is described in detail below. A modification of this method was used by Grinnan and Mosher for the preparation of highly polymerized PNA from rat and rabbit liver.[41] The nucleotide composition of their preparations was not determined. Recently, the preparation of PNA from liver, pancreas, and tumor tissue by the use of sodium dodecyl sulfate has been described by Kay and Dounce.[26]

(1) *Preparation of Mammalian Tissue Ribonucleic Acid.*[25] The method of isolation of ribonucleic acid from tissue homogenates consisted of (a) the removal of deoxyribonucleic acid as a nucleic acid – protein complex, (b) the precipitation of the ribonucleic acid from a cold 2 *M* guanidine hydrochloride solution in which the large bulk of protein remains soluble, and (c) further purification of the ribonucleic acid by chloroform extraction and alcohol precipitations.

The possibility of the occurrence of nuclease action on ribonucleic acid during the preliminary steps of the preparation can be obviated by immediately homogenizing the tissue in concentrated guanidine hydrochloride The latter reagent is an effective protein denaturant. These procedures were found to be applicable to a number of mammalian tissues; details of the methods of preparation follow.

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[44] E. Chargaff, B. Magasanik, E. Vischer, C. Green, R. Doniger, and D. Elson, *J. Biol. Chem.* **186,** 51 (1950).

[45] K. K. Tsuboi and R. E. Stowell, *Biochim. et Biophys. Acta* **6,** 192 (1950).

[46] J. N. Davidson, S. C. Frazer, and W. C. Hutchinson, *Biochem. J.* **49,** 311 (1951).

[47] J. N. Davidson and R. M. S. Smellie, *Biochem. J.* **52,** 600 (1952).

Fresh or frozen tissue was cut in small pieces and blended for 6 to 8 minutes with 3 vol. per gram of tissue of a 0.15 *M* sodium chloride–0.02 *M* phosphate buffer, pH 6.8. A few drops of octyl alcohol were added to reduce foaming. The homogenate was then centrifuged at 3000 *g* for 30 minutes. Essentially all the deoxyribonucleic acid was removed in the form of an insoluble nucleic acid–protein complex as described by Mirsky and Pollister.[12] All operations were carried out between 2 and 5°.

To the supernatant solution enough solid guanidine hydrochloride was added, with rapid stirring, to make the solution 2 *M* with respect to guanidine hydrochloride. The solution was placed in a 38° bath and allowed to stand at this temperature for 30 minutes, then chilled at 0° for 1 hour. Under these conditions most of the protein of the tissue extract remained soluble, while a gelatinous precipitate formed which contained ribonucleic acid and a small amount of protein. The precipitate was washed twice with a cold solution of 2 *M* guanidine hydrochloride (1 vol. per gram of original tissue) and extracted with chloroform–octyl alcohol (5:1). The suspension of nucleic acid in guanidine hydrochloride was added to an equal volume of the chloroform–octyl alcohol mixture, warmed to 40°, then shaken mechanically for 30 minutes. The mixture was centrifuged and the upper aqueous layer containing the nucleic acid removed. The extraction of the aqueous solution at 40° was repeated twice with fresh chloroform–octyl alcohol. Extractions in the cold, or in saline or water solutions, resulted in incomplete separation of the nucleic acid from protein. Nucleic acid was precipitated in the cold from the guanidine solution by adjusting the acidity to pH 4.2–4.5 with acetic acid and adding 2 vol. of cold ethanol. The white, flocculent ribonucleic acid precipitate was centrifuged and washed twice with cold 70% alcohol. The precipitate was then dissolved in water, carefully adjusted to pH 6.8 with dilute sodium hydroxide, and any insoluble material (denatured protein) centrifuged off. The ribonucleic acid was purified by adding enough 1 *M* sodium chloride to bring the final concentration to 0.05 *M* sodium chloride and precipitating the sodium ribonucleate with 2 vol. of cold ethanol. The product was washed twice with cold 70% ethanol.

In the second method the tissue was immediately homogenized with 3 vol. per gram of tissue of cold 2.5 *M* guanidine hydrochloride solution. The rest of the procedure followed that of the first method, except that the ribonucleic acid–protein complex was washed at least three times with cold 2 *M* guanidine hydrochloride to ensure complete removal of any contaminating DNA. Excess foaming, which occurred during the blending in the presence of guanidine hydrochloride, was alleviated by adding a few drops of octyl alcohol after the solution had warmed a few minutes in the 38° bath.

Duplicate liver ribonucleic acid preparations made by the two methods had an essentially identical analytical composition, indicating that in liver little or no enzymatic hydrolysis occurred in the first procedure.

The mammalian ribonucleic acids readily dissolved in water to give clear, colorless solutions. Preparations to be stored were lyophilized from water solutions. Concentrations as high as 20 mg. per cubic centimeter failed to give a reaction with diphenylamine reagent, indicating that all the nucleic acid was of the ribose type.

Similar concentrations gave negative biuret tests. The yield of ribonucleic acids varied from 20 to 30% of the total tissue ribonucleic acid.

3. ISOLATION OF PNA FROM THE TISSUES OF HIGHER PLANTS

The study of the nucleic acids of plant tissues has been rather neglected in recent years. Kay and Dounce[26] reported the isolation of PNA from wheat germ by a method analogous to that used for animal tissue, namely by the use of sodium dodecyl sulfate. Their product is characterized by a very low ratio of nitrogen to phosphorus and may be contaminated with other phosphorus-containing compounds. So far, no analyses of the nucleotide composition of preparations of plant PNA have been reported. On the other hand, the nucleic acids of plant viruses have been isolated and their composition and properties have been determined. The pentose nucleic acids of several strains of tobacco mosaic virus and the related rib grass viruses, were detached from protein by heating for 1 minute as described by Cohen and Stanley,[27] and modified by Knight.[19] In the case of turnip yellow mosaic virus, the protein was denatured and the PNA liberated by treatment with 30% ethanol.[48]

(1) *Isolation of PNA from Strains of Tobacco Mosaic Virus and Cucumber Virus.*[19] Six cubic centimeters of 0.13 *M* sodium chloride, 0.001 *M* with respect to Sörensen's phosphate (K_2HPO_4–KH_2PO_4, 11:5) and at a pH of about 7, was heated in a 15-cc. conical glass centrifuge tube in a water bath at 100°. To this was added 2 cc. of virus solution at a concentration of 30 to 80 mg. of virus per cubic centimeter. The mixture was stirred by being drawn up and down in a dropping pipet for about 15 seconds. By this time the mixture had reached a temperature of about 100° and heating was continued for 1 minute; the tube was then withdrawn and placed in an ice bath. The contents of several tubes were usually pooled and spun at 7000 r.p.m. in an angle centrifuge in order to remove coagulated protein. The clear supernatant fluid containing the sodium nucleate was dialyzed overnight at 4° against 18 l. of flowing distilled water in a Kunitz-Simms rocking dialyzer. The ultraviolet absorption at 260 mμ of the nucleate before and after dialysis was essentially identical, which indicates that no significant quantity of dialyzable nucleic acid material was produced by the cleavage method or by subsequent dialysis. The dialyzed nucleate solution was concentrated to 0.07 to 0.02 of its volume by pervaporation, and the small amount of insoluble matter appearing during concentration, together with small quantities of soluble virus fragments, removed by centrifugation at 40,000 r.p.m. (102,000 × *g*, average) for 1 hour in the No. 40.2 rotor of the Spinco model L centrifuge. The clear supernatant fluid was lyophilized and the residue was dried to constant weight in a drying oven at 110° or *in vacuo* over P_2O_5 at 78°.

Various modifications of the above procedure were tried with results which may be summarized as follows. Lithium chloride can be substituted for sodium chloride. If the final concentration of salt is about 0.1 *M*, coagulation of the denatured protein seems to be greatly aided by the presence of a small amount of phosphate, but, if a salt concentration in the neighborhood of 0.3 *M* is used, the phosphate is dispensable. In tests with up to 1 *M* of sodium chloride, the yields of sodium nucleate were found to diminish above 0.3 M, owing probably to the rapid coagulation of virus befor cleavage of the nucleic acid. Presumably for the same reason, salts of polyvalen metals, such as magnesium or aluminum chloride, gave low yields of nucleate. Heat ing times from 15 seconds up to 10 minutes at 100° were investigated and it was foun that 1 minute was optimum, the yield of nucleate being virtually quantitative at thi point. The yield of nucleate was greatly reduced when, owing to the size and shape o the reaction vessel or to a diminution of the heating, the temperature within th reaction mixture failed to rise above 99°. Hence, the optimum temperature seems t be about 100°, although temperatures higher than 100° were not studied, and th critical temperature between 96–100° was not ascertained. The concentration of viru in the heated mixture affected the cleavage, as previously noted by Cohen and Stan ley.[27] Moreover, when the concentration was high, there appeared to be more nucleo protein in the final preparation than when the final virus concentration did not excee 20 mg. per cubic centimeter of salt–virus mixture.

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[48] R. Markham and J. D. Smith, *Biochem. J.* **49,** 401 (1951).

4. Isolation of PNA from Microbial Tissues

PNA was first isolated from yeast, and yeast is still the preferred starting material for PNA preparations. However, in most procedures for the isolation of yeast PNA alkali is used, and the preparations are of low molecular weight and have lost a portion of their pyrimidine nucleotides It is questionable whether any of the procedures described so far for the isolation of PNA from yeast can result in the production of a preparation that has escaped major degradation. One reason for the failure to obtain better preparations from a material as rich in PNA as yeast, is the difficulty of breaking the wall of the yeast cell. Unless the cell is dried by extraction with organic solvents such as ethanol or acetone, it is impossible to isolate the major portion of the nucleoproteins. This rough treatment is in all likelihood responsible for an extensive degradation of the PNA. The nucleoproteins may be extracted with saline, purified, and fractionated by isoelectric precipitation, and PNA prepared from the nucleoprotein fractions by treatment with 10% NaCl.[23,49] Another method consists of grinding the defatted yeast in a bacterial mill, followed by extraction of the PNA with 10% NaCl. This method is given in detail below.[44] Loring and his collaborators used short treatment with dilute alkali in the cold to extract PNA from yeast.[50]

Little is known about bacterial PNA. Bernheimer[51] was able to show that pentose nucleic acids from *Streptococcus pyogenes*, *Clostridium welchii*, and *Escherichia coli* were inhibitors of group A streptococcal deoxyribonuclease; PNA preparations from mammalian tissues, wheat germ, and yeast failed to show inhibition, while tobacco mosaic virus PNA was inhibitory, but only in relatively high concentrations.

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[49] Y. Khouvine and H. De Robichon-Szulmajster, *Bull. soc. chim. biol.* **34,** 1056 (1952).

[50] H. S. Loring, J. L. Fairley, and H. L. Seagran, *J. Biol. Chem.* **197,** 823 (1952).

[51] A. W. Bernheimer, *Biochem. J.* **53,** 53 (1953).

The isolation of pure PNA is made difficult by the presence of large amounts of DNA in bacterial cells. This difficulty seems to have been overcome in a recently described procedure where a mixture of DNA and PNA was extracted from disintegrated cells of *Mycobacterium tuberculosis*, *Mycobacterium phlei*, and of *Sarcina lutea*, and precipitated with cetyltrimethylammonium bromide.[51a] PNA could be separated from DNA by fractionation of the cetyltrimethylammonium salts with 0.5 M sodium chloride at 0° C.[51b] In a second method PNA was preferentially adsorbed on charcoal from 0.14 M sodium chloride[52] and subsequently eluted by means of 15% phenol at pH 7–7.5.[51b] Very recently the isolation of PNA of *Mycobacterium phlei* by extraction with 5% NaCl and precipitation by acid has been described in a short communication.[53]

(1) *Preparation of PNA from Yeast.*[44] The preparation was made from freshly ground, defatted baker's yeast. The yeast cells (95 g.) were washed with 0.14 M NaCl, and then with 50, 75, 95, and 100% ethanol. Their suspension in equal volumes of 0.14 M NaCl and absolute alcohol was passed through an ice-cooled wet crushing mill for bacteria and 2 vol. (240 cc.) of 70% ethanol was added to the mixture. The precipitate of mostly crushed cells was washed repeatedly with 80 and 90% ethanol, ethanol–ether (1:1), and ether, and dried *in vacuo*. It was twice extracted with 100-cc. portions of 10% aqueous sodium chloride at 90° for ½ hour, and 2 vol. of ethanol was added to the combined centrifuged extracts. The resulting precipitate, washed with dilute and absolute alcohol and ether and dried, weighed 0.71 g. It was taken up in 35 cc. of water, the mixture was centrifuged, and 0.25 vol. of 20% barium acetate solution (pH 7) and 1 vol. of ethanol were added to the supernatant. The precipitate resulting from the centrifugation of the chilled mixture was washed with 5% barium acetate and its aqueous suspension (17 cc.) stirred in a high-speed mixer in the presence of a small excess (150 mg.) of sodium sulfate. The solution, clarified by centrifugation, was freed of protein by being stirred six times in a high-speed mixer with chloroform–octanol (9:1) and then was poured into 2½ vol. of ice-cold ethanol (50 cc.) that was made 0.05 N with respect to HCl. The mixture was chilled overnight and the precipitate, after being washed with alcohol, was suspended in 20 cc. of water and brought into solution by the cautious addition of dilute ammonia to pH 6. The precipitation with acidified alcohol was repeated and the nucleic acid washed with 80 and 100% alcohol and ether and dried, when 0.17 g. of an almost white powder was obtained.

V. The Nature of PNA

The final step in the preparation of PNA consists in the precipitation of the material with ethanol at pH 7.0, 4.2, or 1. Consequently, PNA is obtained as sodium nucleate, acid sodium nucleate, or free PNA. Recent procedures have favored the isolation as sodium nucleate.[54] In this way

[51a] A. S. Jones, *Biochim. et Biophys. Acta* **10,** 607 (1953).

[51b] S. K. Dutta, A. S. Jones, and M. Stacey, *Biochim. et Biophys. Acta* **10,** 613 (1953).

[52] S. Zamenhof and E. Chargaff, *Nature* **168,** 604 (1951).

[53] Y. Khouvine, M. Barbier, and L. Wyssmann, *Compt. rend.* **236,** 2118 (1953).

[54] B. Magasanik and E. Chargaff, *Biochim. et Biophys. Acta* **7,** 396 (1951).

the possible degradation of the material by acid can be avoided. Moreover, sodium nucleate dissolves in water to give a clear, colorless solution whereas free PNA is almost insoluble.

The purity and homogeneity of PNA preparations can be determined by physical and chemical methods. The former are discussed in Chapters 13 and 14; so far they have not been used to any great extent for the characterization of PNA. The behavior of a number of PNA preparations in the ultracentrifuge has been recorded. Cohen and Stanley observed that PNA, freshly isolated from tobacco mosaic virus, sedimented from a 1.0% solution in 0.2 *M* NaCl at pH 4.9 with a constant $S_{20,\mathrm{w}}$ of 5.9.[27] The material was found to be inhomogeneous. Similarly high sedimentation constants were obtained by Grinnan and Mosher[41] with a preparation of rat liver PNA, and by Kay and Dounce with rabbit liver PNA.[26] Comparison of these results is difficult as different concentrations of PNA as well as solvents of different ionic strength and pH were used. The importance of the composition of the solvent is shown by the observations of Grinnan and Mosher: their preparation was polydisperse in sodium chloride solution but monodisperse in water. Volkin and Carter[25] observed a doubling of the sedimentation constant of a preparation of rabbit liver PNA when the pH was lowered from 6.7 to 4.8. At the higher pH, several of their preparations from different animal organs appeared to sediment as a single boundary with $S_{20,\mathrm{w}}$ of about 2.3.

The behavior of liver PNA, yeast PNA, and pancreas PNA on dialysis has been studied by Magasanik and Chargaff[54] and by Kerr and Seraidarian.[13] In all cases the isolated PNA preparations contained dialyzable fractions amounting to 10–25% of the preparation. The dialyzable material consisted of polynucleotides. In the case of yeast PNA the composition of this fraction did not differ materially from that of the undialyzable material, while in the case of the animal PNA a marked difference was observed.[54]

These few observations indicate that PNA preparations are far from homogeneous. The lack of homogeneity of these preparations may be due in part to their decomposition on storage. The stability of PNA has not been carefully investigated, but isolated observations indicate that they are indeed very labile substances. Some preparations cannot be stored even in the cold. Thus, freshly isolated PNA of tobacco mosaic virus (Nucleate A) whose molecular weight was estimated at 300,000, spontaneously decomposed at 4° and pH 7.0 in less than one week to give material with a molecular weight of approximately 60,000.[27] Similarly, the viscosity of freshly prepared solutions of highly polymerized rat liver PNA decreased with time, indicating spontaneous degradation.[41] Beef pancreas PNA isolated by Kerr and Seraidarian could not be dried without partial decomposition.[13] It has been mentioned before that this pancreas PNA

TABLE II

THE ULTRAVIOLET EXTINCTION AT 260 mμ OF PNA

Preparation	$\epsilon(P)$	$\epsilon(P)$ after alkaline hydrolysis	Increase, %	Ref.
Mouse liver	8,190	10,700	31	45
Pig liver	8,600	11,100	29	54
Calf pancreas	7,750	9,570	24	26
Yeast	10,000	12,400	24	54
Yeast	8,700	11,900	37	54

preparation contained nucleotide fragments which could be removed by dialysis in the cold. Continuation of the dialysis at room temperature resulted in the appearance of more polynucleotides in the dialysate, indicating lack of stability at room temperature.

The extinction of ultraviolet light is a characteristic property of nucleic acids. [Cf. *Beaven*, *Holiday*, *and Johnson*, Chapter 14.] The chromophores are the conjugated double bond systems of the purines and pyrimidines. The ultraviolet extinctions of nucleic acids and nucleotides are conveniently expressed as $\epsilon(P)$, the extinction of a solution containing one gram-atom of nucleotide phosphorus per liter.[55] The $\epsilon(P)$ values for a number of different PNA preparations are summarized in Table II. It can be seen that the ultraviolet extinction of the PNA preparations is less than the sum of the extinctions of the mononucleotides to which they can be hydrolyzed by alkali. It seems that the polymerization of mononucleotides to form PNA is accompanied by the suppression of certain chromophores. The level of polymerization at which this effect becomes noticeable cannot be defined. It is not due to the simple union of a few mononucleotides, since the dialyzable polynucleotides show the same extinction as the mixture to which they can be hydrolyzed by alkali. On the other hand, the highly polymerized PNA preparations obtained by the use of sodium dodecyl sulfate do not show a greater degree of suppression of extinction than the less highly polymerized preparations obtained by salt extraction. Still, the measurements of the increase in ultraviolet absorption obtained by exposure of PNA to alkali would appear to be of value in the characterization of such preparations.

The chemical methods used to characterize PNA include the estimation of the nucleotide composition, which will be discussed separately, the determination of nitrogen, of total and acid-hydrolyzable phosphorus, and of pentose. The nitrogen and total phosphorus content differs with the nature of the sample. Free PNA contains about 15–16% nitrogen and 8.5–9% phosphorus, while the values for sodium nucleates range around

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[55] E. Chargaff and S. Zamenhof, *J. Biol. Chem.* **173,** 327 (1948).

12% and 8%, respectively. More important than the actual values is th atomic N/P ratio. A nucleic acid composed of equimolar amounts of th four nucleotides would have an N/P ratio of 3.75. Preponderance of purine over pyrimidines and of cytosine over uracil results in an increased N/ ratio. PNA preparations from animal sources, particularly from pancreas have N/P ratios as high as 4.3. N/P ratios higher than this are indicativ of contamination of the preparation with other nitrogenous substances generally with protein.

The amount of inorganic phosphorus obtained after hydrolysis of PN with dilute acid is a measure of the purine content of the preparation, fo only purine-nucleoside-bound phosphate is hydrolyzed under these conditions. Similarly, the estimation of pentose by the orcinol reaction is a measure of purine-bound pentose. Pyrimidine nucleotides are not hydrolyzed under the conditions of this test, and consequently, the pyrimidine-bound pentose does not react with orcinol. [Cf. *Dische*, Chapter 9.]

It is usual to measure the amount of protein and DNA in the PNA preparations by specific methods, such as the biuret test, and the diphenylamine reaction, respectively. The PNA preparations which are obtained by the isolation methods presented above are usually free from protein, and do not contain more than 5% DNA. In general, contamination with DNA is more effectively excluded when the nuclei are removed prior to the isolation of PNA. The presence of DNA does not interfere with the estimation of pentose nucleotides by paper chromatography or ionophoresis. [Cf. Chapters 7 and 8.]

The colorimetric estimation of pentoses by the orcinol method does not differentiate between ribose and other pentoses. However, the nature of the sugar component may be determined after hydrolysis by isolation and conversion into derivatives, or by paper chromatography in various solvents. By these methods the pentose components of PNA preparations isolated from mammalian organs,[4,24,44] from tobacco mosaic virus,[56,57] from cucumber virus CV3,[57] and from *C. perfringens*,[22] have been unequivocally identified as D-ribose. At present, pentose nucleic acids isolated from these sources may safely be called ribonucleic acids.

VI. The Nucleotide Composition of PNA

1. General Considerations

The analysis of purified DNA preparations isolated from a great variety of cells has led to the conclusion that (a) the deoxypentose nucleic acids of different species of organisms have different nucleotide compositions, (b) those of different organs of the same species have identical compositions, and (c) the ratios of adenine to thymine and guanine to cytosine

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[56] R. Markham and J. D. Smith, *Biochem. J.* **46,** 513 (1950).

[57] D. L. MacDonald and C. A. Knight, *J. Biol. Chem.* **202,** 45 (1953).

including methylcytosine) are always unity.[58] The implications of these ndings are discussed in Chapter 10 and elsewhere in this book. Here the esults obtained by the analysis of PNA preparations will be examined ith the view to finding out whether similar or different generalizations an be made with regard to the composition of PNA.

2. Analytical Procedures

The methods used for the estimation of nucleic acid components have een discussed in Chapters 5 to 9. Here only a brief description will be iven of those methods which served to obtain most of the results presented in the succeeding sections.

Method 1.[59] Hydrolysis of PNA by N HCl to a mixture of purine bases and pyrimiine mononucleotides followed by separation of the components of the mixture by aper chromatography, elution, and spectrophotometric estimation.

Method 2.[60, 44] Hydrolysis of PNA by dilute alkali to a mixture of mononucleotides, eparation of the mixture by paper chromatography, followed by elution and specrophotometric estimation. In this method buffered isobutyric acid is used as the rganic phase in chromatography. Guanylic acid and uridylic acid, which occupy the ame position on the chromatogram, are eluted together and their respective concenrations determined from the extinction values of the mixed eluate at two different avelengths by means of simultaneous equations. The value for the molecular extincion of guanylic acid in M phosphate buffer of pH 7.0 which was used by the authors o calculate the concentration of guanylic acid and of uridylic acid was obtained rom measurements carried out on an impure sample of sodium guanylate and corected according to the nitrogen content of this sample. Recent determinations of he molecular extinction of guanylic acid purified by ion-exchange chromatography ave shown that the value used was too high. [Cf. Chapter 14.] Consequently the esults of the analysis of pentose nucleic acids by Chargaff *et al.*[44] and by Magasanik nd Chargaff[54] report values for guanylic acid which are too low. The compositions f these preparations have been recalculated using the following extinction coeffiients taken from a recent paper of Elson *et al.*[61] ($\Delta\epsilon$ is the difference between the olecular extinction at the wavelengths indicated and that at 290 μ.)

Nucleotide	$\lambda, m\mu$	$\Delta\epsilon$
denylic acid	260	15.12
uanylic acid	265	7.18
	252.5	10.74
	245	8.87
ytidylic acid	270	6.87
ridylic acid	265	9.49
	261	9.80
	245	5.47

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[58] E. Chargaff, *Experientia* **6,** 201 (1950); *Federation Proc.* **10,** 654 (1951).

[59] J. D. Smith and R. Markham, *Biochem. J.* **46,** 509 (1950).

[60] B. Magasanik, E. Vischer, R. Doniger, D. Elson, and E. Chargaff, *J. Biol. Chem.* **186,** 37 (1950).

[61] D. Elson, T. Gustafson, and E. Chargaff, *J. Biol. Chem.* **209,** 285 (1954).

The results reported in the subsequent sections were calculated using these values.

Method 3.[25] Hydrolysis of PNA by dilute alkali to a mixture of mononucleotides, separation of the mixture by chromatography on a basic ion-exchange resin (Dowex 1) [compare *Cohn*, Chapter 6], elution with acid, and spectrophotometric estimation. In this method the 2′- and 3′-phosphates of the purine nucleosides, which are formed by the action of alkali, are separated. The results recorded here give the sum of the 2′- and 3′-phosphates of adenine or guanine.

Method 4.[62, 63] Hydrolysis of PNA by dilute alkali, followed by separation of the mixture of mononucleotides by electrophoresis on filter paper, elution, and spectrophotometric estimation or phosphorus analysis. [Cf. *Smith*, Chapter 8.]

Method 5.[64, 65] Hydrolysis of PNA by N H_2SO_4 to a mixture of purines and pyrimidine nucleotides. The purines are precipitated as insoluble silver salts; these are decomposed by hydrochloric acid, and the concentration of adenine and of guanine in the supernatant solution determined by measurement of its ultraviolet extinction at two wavelengths. The concentrations of the pyrimidine nucleotides in the supernatant solution of the purine silver salts are similarly determined. Alternatively the pyrimidine nucleotides may be converted to nucleosides by treatment with prostatic phosphatase prior to spectrophotometric estimation. [Compare also *Loring*, Chapter 5.]

Methods 1, 3, and 4 permit the separation of the four nucleic acid components and are therefore presumably superior in accuracy to methods 2 and 5. The recovery of pyrimidine nucleotides in method 1 may be low, even when the correction described by the authors is applied.[63]

In general the reliability of a method is judged by the completeness with which the sum of the products obtained will account for the phosphorus and nitrogen content of the PNA preparation. The methods described allow 90–100% of the nitrogen and the phosphorus content to be accounted for as bases or nucleotides. Usually the composition of the PNA sample is presented as the fraction of PNA phosphorus accounted for in each nucleotide. However, in order to facilitate the comparison of the composition of different PNA preparations it is advantageous to express the results as the molar ratios of the nucleotides relative to adenine as 10.[44] It is in this manner that the composition of PNA preparations is presented in the succeeding sections.

3. PNA from Animal Tissues

A sufficiently large number of animal PNA preparations has been isolated and analyzed to permit consideration of the question whether animal pentose nucleic acids are species-specific or organ-specific or fall into no clearly discernible pattern. The composition of PNA preparations isolated

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[62] J. N. Davidson and R. M. S. Smellie, *Biochem. J.* **52,** 594 (1952).

[63] G. W. Crosbie, R. M. S. Smellie, and J. N. Davidson, *Biochem. J.* **54,** 287 (1953).

[64] S. E. Kerr, K. Seraidarian, and M. Wargon, *J. Biol. Chem.* **181,** 761 (1949).

[65] H. S. Loring, J. L. Fairley, H. W. Bortner, and H. L. Seagran, *J. Biol. Chem.* **197,** 809 (1952).

TABLE III

Nucleotide Composition of PNA Isolated from Liver

Preparation	Animal	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Pu/Py	Method[a]	Ref.
1	Rabbit	10	16.9	14.6	10.3	1.08	4	63
2	Rabbit	10	19.7				4	63
3	Rabbit	10	17.0				4	63
4	Rabbit	10	16.5				4	63
5	Rabbit	10	15.4				4	63
6	Rabbit (pregnant)	10	15.8	15.2	11.1	0.98	4	63
7	Rabbit (fetal)	10	15.4	15.6	10.0	0.99	4	63
8	Rabbit	10	20.2	16.8	9.9	1.13	3	25
9	Rabbit	10	19.4	16.1	9.5	1.12	3	25
10	Rat	10	17.5	13.9	10.9	1.10	4	63
11	Rat	10	17.6	14.3	10.8	1.10	4	63
12	Rat (regenerating)	10	16.6	14.1	10.2	1.08	4	63
13	Rat	10	18.3	18.9	8.5	1.04	3	25
14	Rat (regenerating)	10	19.0	18.3	9.3	1.05	3	25
15	Beef	10	17.0	10.4	7.8	1.49	2	44
16	Beef (calf)	10	18.8	10.8	6.7	1.65	2	44
17	Beef (calf)	10	17.9	14.9	8.4	1.20	3	25
18	Beef (calf)	10	18.7	16.0	8.7	1.17	3	25
19	Chicken	10	17.1	13.6	10.6	1.12	4	63
20	Mouse	10	16.2	12.9	8.6	1.22	3	25
21	Sheep	10	19.4	12.7	7.1	1.49	2	44
22	Pig	10	18.8	15.3	9.1	1.23	2	44
23	Man	10	38.6	27.5	11.0	1.26	2	44

[a] See page 395.

from the liver of a variety of species of animals is presented in Table III and will be considered first. It can be seen that the liver PNA preparations of all the animals studied are rich in guanylic acid and cytidylic acid, and poor in adenylic acid and uridylic acid. The ratio of purines to pyrimidines is generally not far from unity. The preparations exhibit considerable variation in nucleotide ratios, but no striking differences in the composition of liver PNA of different animals indicative of species-specificity can

TABLE IV

NUCLEOTIDE COMPOSITION OF PNA ISOLATED FROM DIFFERENT ORGANS OF CALF

Preparation	Organ	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Pu/Py	Method[a]	Ref.
1	Liver	10	17.9	14.9	8.4	1.20	3	25
2	Pancreas	10	34.5	16.8	9.5	1.69	3	25
3	Spleen	10	19.7	17.7	8.6	1.13	3	25
4	Thymus	10	23.8	13.9	6.5	1.65	3	25

[a] See page 395.

be discovered. Some of the observed differences are undoubtedly due to differences in the method of isolation. Thus different values are reported for the cytidylic acid content of calf liver PNA isolated in different laboratories (preparations 16 and 17). Of greater interest are the differences in the composition of PNA preparations isolated by the same method. A large number of preparations were isolated from the livers of different rabbits and rats by Davidson and his collaborators.[63] The individual variations in the composition of liver PNA of different rabbits (preparations 1–7) were found to be greater than the differences between the average composition of rabbit liver PNA and rat liver PNA. The only PNA preparation which differs significantly from all the others in composition is that isolated from a human liver (preparation 23). It seems, however, unwarranted to attach significance to an isolated observation.

In Table IV the composition of PNA preparations obtained from different organs of the same animal are compared. Calf spleen PNA can be seen to differ little from liver PNA, whereas thymus and particularly pancreas PNA are considerably richer in guanylic acid. In consequence, the purine-to-pyrimidine ratios of calf thymus PNA and of pancreas PNA approach 2.0. The high guanylic acid content of pancreas PNA has also been observed in other laboratories. However, the preparations isolated by different procedures vary greatly in composition (Table V). This variation may partly be ascribed to the extent of degradation by pancreatic ribonuclease which PNA undergoes during the course of the isolation procedure. The influence of the action of the enzyme on the composition of the final product is clearly demonstrated by comparison of preparations 3 and 4 in Table V. The former was prepared in the usual manner, while the latter was isolated from the tissue after removal of ribonuclease by extraction with dilute acid and acetone;[38] preparation 4 is considerably richer in pyrimidines and seems to be essentially identical in composition with liver PNA.

The action of pancreatic ribonuclease on PNA preparations isolated

TABLE V

Nucleotide Composition of PNA Isolated from Pancreas

<table>
<tr><th>Preparation</th><th>Animal</th><th>Adenylic acid</th><th>Guanylic acid</th><th>Cytidylic acid</th><th>Uridylic acid</th><th>Pu/Py</th><th>Method[a]</th><th>Ref.</th></tr>
<tr><td>1</td><td>Beef</td><td>10</td><td>42</td><td>32</td><td>10</td><td>1.2</td><td>5</td><td>65a</td></tr>
<tr><td>2</td><td>Beef (calf)</td><td>10</td><td>34.5</td><td>16.8</td><td>9.5</td><td>1.7</td><td>3</td><td>25</td></tr>
<tr><td>3[b]</td><td>Beef</td><td>10</td><td>21</td><td colspan="2">15</td><td>2.0</td><td>[d]</td><td>38</td></tr>
<tr><td>4[c]</td><td>Beef</td><td>10</td><td>18</td><td colspan="2">30</td><td>1.0</td><td>[d]</td><td>38</td></tr>
<tr><td>5</td><td>Pig</td><td>10</td><td>26.7</td><td>9.3</td><td>4.9</td><td>2.6</td><td>2</td><td>44</td></tr>
</table>

[a] See page 395.
[b] Prepared in usual way.
[c] Ribonuclease removed prior to isolation.
[d] Indirect colorimetric methods.

TABLE VI

Nucleotide Composition of PNA "Cores" Resistant to Pancreatic Ribonuclease

<table>
<tr><th>Preparation</th><th>Tissue</th><th>Adenylic acid</th><th>Guanylic acid</th><th>Cytidylic acid</th><th>Uridylic acid</th><th>Pu/Py</th><th>Method[a]</th><th>Ref.</th></tr>
<tr><td>1</td><td>Beef pancreas</td><td>10</td><td>23</td><td colspan="2">15</td><td>2.2</td><td>[b]</td><td>38</td></tr>
<tr><td>2</td><td>Pig liver</td><td>10</td><td>49.1</td><td>8.2</td><td>6.7</td><td>4.0</td><td>2</td><td>54</td></tr>
<tr><td>3</td><td>Yeast</td><td>10</td><td>43</td><td>4</td><td>7</td><td>4.8</td><td>2</td><td>54</td></tr>
<tr><td>4</td><td>Yeast</td><td>10</td><td>14</td><td colspan="2">10</td><td>2.4</td><td>[b]</td><td>38</td></tr>
</table>

[a] See page 395.
[b] Indirect colorimetric methods.

from liver or from yeast results in the formation of pyrimidine mononucleotides and of polynucleotides of different sizes consisting mostly of purines [see Chapter 15]. The largest of these polynucleotides, the so-called "core," is very rich in guanylic acid and poor in pyrimidine nucleotides (Table VI). Liver PNA is thus transformed by the action of pancreatic ribonuclease into polynucleotides similar in composition to pancreas PNA. It is therefore quite possible that "native" pancreas PNA is of the same composition as liver PNA, but that the action of the ribonuclease converts this native PNA during the isolation procedure into a degraded product of high guanylic acid and low pyrimidine nucleotide content.

[65a] S. E. Kerr, K. Seraidarian, and M. Wargon, *J. Biol. Chem.* **181,** 773 (1949).

TABLE VII

NUCLEOTIDE COMPOSITION OF PNA FROM MISCELLANEOUS ANIMAL SOURCES

Preparation	Tissue	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Pu/Py	Method[a]	Ref.
1	Cat brain	10	14.7	12.0	9.5	1.15	2	66
2	Carp muscle (Nucleotropomyosin)	10	21	19	11	1.0	1	29
3	Sea urchin eggs	10	13.3	12.3	9.3	1.07	2	61
4	Starfish eggs	10	15	14	11	1.0	1	8

[a] See page 395.

Table VII presents the composition of PNA preparations isolated fron miscellaneous animal tissues. It is of interest that the PNA of cat brain[6 and of carp muscle tropomyosin[29] have essentially the same compositior as liver PNA. The PNA of starfish[8] and of sea urchin[61,67] eggs similarl possess the "high guanylic acid, high cytidylic acid" pattern found in al other PNA preparations isolated from the tissues of animals. The composition of the sea urchin egg PNA did not change after fertilization durin the course of 48 hours of embryonic development.[61,67]

The problem of the heterogeneity of PNA in different cell fractions ha been studied in several laboratories.[63,67-69] The cell nuclei were isolated i citric acid or by centrifugation of a tissue mince at 700 × g. The element of the cytoplasm were fractionated by centrifugation (see Chapter 21) PNA was not isolated, but the total polyribonucleotide composition of th fractions determined directly. Some of the results obtained in these studie are presented in Table VIII. There are no significant differences in nucleotide composition between the different cytoplasmic fractions, which, however, appear to differ from the nuclear fractions. The familiar "high guanylic acid, high cytidylic acid" pattern of animal PNA was found i every case. However, the nuclear PNA composition of different animals of the same species seemed to vary appreciably. For instance, the composition of preparation 1, which is the average of the nuclei obtained from the livers of four rats, contains more guanylic acid than most of the other preparations from nuclei. Even higher values for guanylic acid, which would make the composition of nuclear PNA more closely similar to that of cytoplasmic PNA, were found by McIndoe in many batches of nuclei (unpublished results quoted by Crosbie *et al.*).[63] The composition of nuclear

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[66] H. A. Deluca, R. J. Rossiter, and K. P. Strickland, *Biochem. J.* **55,** 193 (1953).

[67] D. Elson and E. Chargaff, *Phosphorus Metabolism* **2,** 329 (1952).

[68] A. Marshak, *J. Biol. Chem.* **189,** 607 (1951).

[69] W. M. McIndoe and J. N. Davidson, *Brit. J. Cancer* **6,** 200 (1952).

TABLE VIII

NUCLEOTIDE COMPOSITION OF PNA FROM DIFFERENT MORPHOLOGICAL FRACTIONS OF RAT AND RABBIT LIVER

Preparation	Animal	Fraction	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Pu/Py	Method[a]	Ref.
1[b]	Rat	Nuclei	10	14.8	14.3	12.9	0.91	4	69
2	Rat	Nuclei	10	13.4	12.1	11.7	0.98	4	69
3	Rat	Nuclei	10	9.0	11.5	11.1	0.84	4	63
4	Rat	Nuclei	10	12.7	14.6	12.2	0.84	2	67
5	Rat	Nuclei	10	13.0	14.9	11.5	0.87	2	67
6	Rat	Nuclei	10	12.0	18.6	14.8	0.66	2	67
7	Rat	Mitochondria	10	16.9	15.1	11.0	1.03	4	63
8[c]	Rat	Mitochondria	10	17.0	14.2	10.3	1.10	4	63
9	Rat	Microsomes	10	16.9	14.7	10.3	1.08	4	63
10[c]	Rat	Microsomes	10	16.8	15.1	10.4	1.02	4	63
11	Rat	Microsomes	10	17.9	17.1	9.9	1.03	2	67
12	Rat	Microsomes	10	18.2	16.1	9.6	1.10	2	67
13	Rat	Cell sap	10	16.9	14.6	11.3	1.04	4	63
14[c]	Rat	Cell sap	10	17.2	14.7	10.5	1.08	4	63
15	Rat	Cell sap	10	17.3	16.5	9.8	1.04	2	67
16	Rat	Cell sap	10	18.0	15.9	9.2	1.12	2	67
17	Rabbit	Nuclei	10	13.6	13.1	14.0	0.87	4	69
18	Rabbit	Mitochondria	10	15.9	15.6	10.3	1.00	4	63
19	Rabbit	Mitochondria	10	15.4	15.5	10.4	0.98	4	63
20	Rabbit	Microsomes	10	15.6	15.4	10.2	1.00	4	63
21	Rabbit	Microsomes	10	16.1	15.5	10.4	1.00	4	63
22	Rabbit	Cell sap	10	15.4	15.3	10.2	1.00	4	63
23	Rabbit	Cell sap	10	15.4	15.3	9.8	1.00	4	63

[a] See page 395.

[b] Average of four preparations.

[c] Regenerating.

PNA was also studied by Marshak[68] by a method in which PNA wa hydrolyzed to a mixture of bases with hot perchloric acid.[70] This procedu was later found to lead to an incomplete hydrolysis of the pyrimidi nucleotides and to poor recoveries of cytosine and uracil.[63] The use of th method may account in part for the exceptionally low cytosine and urac content of nuclear PNA reported by Marshak.[68]

4. PNA from Microbial Tissues

The composition of yeast PNA has been determined in many laboratories. Most investigators who were intent upon developing methods fo the estimation of the nucleotide composition of PNA used commercia yeast nucleic acid, purified to various degrees, as an object for testin their method. However, it has been mentioned earlier that all commercia yeast PNA preparations have been isolated by extraction with alkali, an consequently are badly degraded. The composition of such preparation cannot be considered to represent yeast PNA. For this reason only th composition of yeast nucleic acid specimens isolated in the laboratory b mild procedures will be considered here.[44,49,50] The composition of fiv preparations of PNA from baker's yeast are shown in Table IX. In al cases the yeast was dried with ethanol before extraction of PNA. It ca be seen that these five preparations are quite similar in composition, although the method of isolation was different, except for preparations and 5. Preparation 6, which was isolated from brewer's yeast seems to b of slightly different composition.[49] The composition of yeast PNA approaches a "statistical tetranucleotide" more closely than that of anima PNA. However, baker's yeast PNA was in all cases found to be somewha richer in guanine than in adenine, and somewhat richer in uracil than i cytosine. It must be kept in mind that all purified preparations of yeas PNA account for only a portion of the PNA originally present in yeast Therefore the good agreement in the results obtained may simply indicat that the same portion of yeast PNA is isolated in the procedures used. A different procedure of drying, such as substituting acetone for ethanol, le to a PNA preparation with a guanine content of 14.3, almost one-thir higher than that of the ethanol-dried preparations.[49] An attempt was mad by Khouvine and her collaborators to investigate the heterogeneity o baker's yeast PNA by separating the PNA-proteins isolated from acetone-ground yeast into several fractions by isoelectric precipitations.[23] Th nucleotide composition of the fractions precipitated at pH 5 and 4.3 closel resembled that of the preparations presented in Table IX, whereas th nucleotide composition of the fraction precipitated at a pH of 2.3 wa richer in guanylic acid.

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[70] A. Marshak and H. J. Vogel, *J. Biol. Chem.* **189**, 597 (1951).

TABLE IX

NUCLEOTIDE COMPOSITION OF PNA FROM MICROORGANISMS

Preparation	Organism	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Pu/Py	Method[a]	Ref.
1	Baker's yeast	10	12.0	8.0	9.8	1.23	2	44
2	Baker's yeast	10	11.9	7.2	11.4	1.17	2	44
3	Baker's yeast	10	10.9	8.5	9.5	1.16	5	50
4	Baker's yeast (Springer Sp5)	10	11.4	7.5	9.0	1.30	1	49
5	Baker's yeast (Koenig-Gist)	10	11.1	7.1	8.9	1.32	1	49
6	Brewer's yeast (Karcher)	10	10.4	9.2	9.6	1.08	1	49
7	*Mycobacterium phlei*	10	15.5	8.5	3.5	2.13	1	53
8	*Serratia marcescens*	10	10.2	8.5	8.3	1.2	2	71
9	*Escherichia coli*	10	10.2	8.5	8.3	1.2	2	71
10	*Clostridium perfringens*	10	11.6	9.4	7.4	1.29	2	22

[a] See page 395.

The only PNA isolated from a bacterial species whose nucleotide composition has so far been determined is that of *Mycobacterium phlei.*[53] The composition of PNA of *Serratia marcescens*[71] and of *Escherichia coli*,[71] and of a nucleoprotein of *C. perfringens*[22] were determined without prior isolation of the nucleic acids. The results of the analyses are shown in Table IX.

5. PNA FROM PLANT VIRUSES

The composition of a large number of plant virus PNA preparations has recently been determined by Knight and his collaborators[19,72,73] and by Markham and Smith.[48] The results are of particular interest since in the case of plant viruses we can accept them as describing the composition of native virus PNA without reservations regarding degradation or fractionation during isolation. The purity of the virus preparations can be ascertained by physical methods, and the nucleotide composition of the isolated PNA was found to agree closely with that of the virus, determined without prior isolation of PNA. The results of these determinations are presented

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[71] D. Elson and E. Chargaff, *Nature* **173,** 1037 (1954).

[72] R. W. Dorner and C. A. Knight, *J. Biol. Chem.* **205,** 959 (1953).

[73] F. L. Black and C. A. Knight, *J. Biol. Chem.* **202,** 51 (1953).

TABLE X

NUCLEOTIDE COMPOSITION OF PNA ISOLATED FROM PLANT VIRUSES

Preparation	Virus	Strain	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Pu/Py	Method[a]	Ref.
1	Tobacco mosaic	TMV	10	8.5	6.2	8.8	1.24	1	19
2	Tobacco mosaic	M	10	8.9	6.5	8.8	1.23	1	19
3	Tobacco mosaic	J14D1	10	8.4	6.2	8.9	1.22	1	19
4	Tobacco mosaic	GA	10	8.9	6.6	8.9	1.22	1	19
5	Tobacco mosaic	YA	10	8.6	6.2	8.9	1.23	1	19
6	Tobacco mosaic	HR	10	8.8	6.2	9.2	1.22	1	19
7	Cucumber	CV3	10	9.9	7.1	11.9	1.05	1	19
8	Cucumber	CV4	10	10	7.5	11.5	1.05	1	19
9	Tomato bushy stunt	BS	10	10	7.4	8.9	1.23	1	72
10	Turnip yellow mosaic	TY	10	7.6	16.8	9.8	0.66	1	48
11	Southern bean mosaic	SBM	10	10	8.9	9.8	1.07	1	72
12	Potato X	PX	10	6.2	6.6	6.2	1.27	1	72

[a] See page 395.

in Table X. It can be seen that different viruses have completely different nucleotide composition. For instance, cytidylic acid varies from 6.6 in potato virus X to 16.8 in turnip yellow mosaic virus. There does not appear to be a regularity comparable to the constant purine-to-pyrimidine ratio of 1, observed in all DNA preparations. [Compare Chapter 10.] The most striking observation made by Knight and his collaborators is that all sixteen strains of tobacco mosaic virus, are identical in composition.[19,73] Similarly, the nucleotide composition of two strains of cucumber virus is identical, but different from that of the closely related tobacco mosaic virus.[19] These results present impressive evidence that the PNA of plant viruses is species-specific, and that strains which have developed by mutation of the parent strain do not differ from it in nucleotide composition. A similar relation was found in the DNA of bacteria: different species possess nucleic acids of different composition, but different strains of the same species yield specimens of the same composition.[74] It has recently been shown that the DNA preparations are composed of a large number of molecules of different composition.[75] The DNA composition of the DNA preparation isolated from an organ or a group of cells represents the average composition of the individuals present in this material. It is quite possible that the viral PNA is similarly composed of a population of molecules of different composition. It would appear then that all strains of the same virus contain these nucleic acids in the same proportions.

The species-specificity of PNA in plant viruses agrees well with the postulate of a genetic function for this PNA. The plant viruses are able to bring about their own production in susceptible cells. Consequently, they must possess a genetic determinant. The lack of DNA, which is considered to be the carrier of heredity in most organisms, suggests that this function is carried out by the PNA present in the virus.

6. Conclusions

The results of the determinations of the nucleotide composition of PNA preparations which have been presented in the preceding sections are too scattered, and even contradictory, to permit any definite statements regarding regularities in PNA composition to be made. However, a few tentative conclusions can be drawn from the results obtained so far, and these may point out the directions for future work.

(1) *PNA may be species-specific*. PNA is clearly species-specific in plant viruses: different viral species possess PNA of characteristically different composition, while different strains of the same species possess PNA of

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[74] B. Gandelman, S. Zamenhof, and E. Chargaff, *Biochim. et Biophys. Acta* **9**, 399 (1952).

[75] E. Chargaff, C. F. Crampton, and R. Lipshitz, *Nature* **172**, 289 (1953).

identical composition. Yeast PNA and animal PNA differ in compositio from one another and from viral PNA. However the variations encountere in the composition of PNA preparations isolated from different animals o the same species are as great as the variations encountered in animals o different species. These individual variations in PNA composition remai unexplained. It is unlikely that they are due to genetic differences, for the greater differences between species would be expected. They do not seem to depend on the metabolic state of the organ from which the PNA i isolated, as preparations isolated from normal and from regenerating ra liver did not differ appreciably in composition. It will be necessary t determine critically the range of variation of PNA composition withi members of the same species before fruitful comparisons of PNA preparations from different species can be made.

(2) *PNA is presumably not organ-specific.* So far no convincing differences in the composition of PNA preparations isolated from different organs of the same animal have been observed. The high guanylic acid and low pyrimidine nucleotide content of pancreas PNA seems to be due to the extensive degradation by pancreatic ribonuclease during isolation, and not to reflect a difference in composition between native PNA from pancreas and from other organs.

(3) *The nuclear and cytoplasmic fractions of cells differ in PNA composition.* PNA isolated from whole organs consists almost exclusively of cytoplasmic PNA, as the nuclei are largely removed during isolation, and a nuclear PNA accounts for only 10% of the total PNA of the cell. [Compare Chapters 18, 19, and 21.] Cytoplasmic PNA does, therefore, not differ appreciably from "whole organ PNA" in composition. The PNA of rat liver nuclei appears to be richer in uridylic acid and poorer in guanylic acid than cytoplasmic PNA. Nuclei isolated from the livers of different rats show greater variation in composition than the corresponding cytoplasmic fractions. Nuclear PNA is known to be more active metabolically than cytoplasmic PNA. The correlation of metabolic activity with changes in nucleotide composition might prove to be of interest.

(4) *The composition of animal, yeast, and bacterial PNA shows certain regularities.* The nucleotide ratios of viral PNA preparations do not fall into an easily discernible pattern. On the other hand, both yeast PNA and PNA of the cytoplasm of animal cells contain purine and pyrimidine nucleotides in nearly equimolar quantities. In animal PNA, guanylic and cytidylic acids predominate, while in yeast PNA the four nucleotides are present in nearly equimolar concentrations. Recently Elson and Chargaff[71] have pointed out that the regularities in the composition of PNA can be demonstrated in a more striking fashion when whole cells or centrifugally prepared cell fractions are subjected to hydrolysis and analysis ensuring

the quantitative recovery of PNA as mononucleotides. In this way changes in nucleotide composition due to the great susceptibility of nucleic acid to enzymic and other degradations during isolation are avoided. These authors were able to show that all pentose nucleic acids analyzed in their laboratory, including specimens from bacteria, yeast, and sea urchin and starfish eggs, as well as from the cytoplasm and the nuclei of vertebrate cells, possessed an equal number of 6-keto (guanine and uracil) and 6-amino (adenine and cytosine) groups. The preparations from animal cytoplasm, marine eggs, and yeast showed the additional regularity of a purine-to-pyrimidine ratio of 1. These relations are well illustrated in the examples taken from the work of these authors as well as from that of others presented in Tables VII, VIII, and IX (preparations 7–10). It is of interest to compare the composition of PNA and DNA of animal cells. PNA is composed of equal amounts of guanylic acid and cytidylic acid, and of adenylic acid and uridylic acid, with the first pair predominating.[71] DNA is composed of equal amounts of guanylic acid and cytidylic acid, and of adenylic acid and thymidylic acid, but the second pair predominates.[58] The composition of DNA is compatible with a double-stranded helical structure, proposed by Watson and Crick on the basis of X-ray diffraction measurements.[76] A similar correlation of physical and chemical properties of PNA has not yet been made.

These are the conclusions which can be drawn from the work of the last seven years, during which modern, accurate methods of nucleotide analysis were used. Further progress will not depend on the development of more accurate and efficient methods of analysis, but rather on new approaches to the problem of the isolation and physical characterization of PNA. Until homogeneous PNA preparations can be isolated, the knowledge of the nucleotide composition of individual PNA specimens will not greatly further the study of the structure and biological role of PNA. The most fruitful approach to the problem of PNA structure has been the study of the degradation products produced enzymically from PNA. The results of such studies, which are discussed in Chapter 15, have shown that animal, yeast, and viral PNA preparations are broken down by ribonuclease into qualitatively very similar mixtures of mono-, di-, and polynucleotides. For example, the largest nucleotide fragments are in all cases extremely rich in guanylic acid. It would thus appear that these PNA preparations, although differing in composition, are constructed according to very similar patterns. The definition of these patterns remains a task for future research.

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[76] J. D. Watson and F. H. C. Crick, *Nature* **171,** 737 (1953).

CHAPTER 12

Evidence on the Nature of the Chemical Bonds in Nucleic Acids

D. M. BROWN AND A. R. TODD

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I. Introduction

Since the emergence of reasonably clear ideas on the nature of the simple nucleotides, and the recognition that the nucleic acids can be regarded as polynucleotides, many attempts have been made to formulate general structures for them. In all such attempts the nature of the internucleotidic linkage has occupied a central position and at one time or another most of the possible types have been considered. The earlier developments in this field have been adequately treated in a number of reviews,[1-6] and since they are primarily of historical interest they will not be discussed here. Fortunately, work in recent years has led to precise and generally accepted views on at least the major portion of the internucleotidic linkages present in both ribonucleic and deoxyribonucleic acids; it is therefore only necessary to

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[1] P. A. Levene and L. W. Bass, "The Nucleic Acids." The Chemical Catalog Co., New York, 1931.

[2] R. S. Tipson, *Advances in Carbohydrate Chem.* **1,** 193 (1945).

[3] J. M. Gulland, *J. Chem. Soc.* **1938,** 1722.

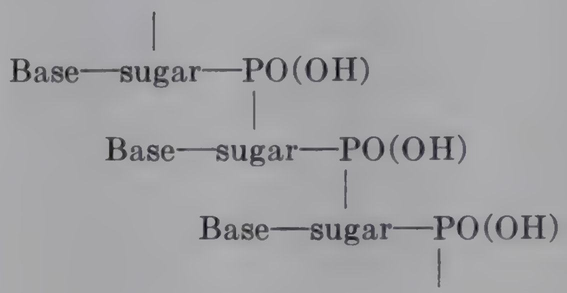
[4] J. M. Gulland, *J. Chem. Soc.* **1944,** 208.

[5] J. M. Gulland, Nucleic acids, *Symposia Soc. Exptl. Biol.* **1,** 1 (1947).

[6] F. Schlenk, *Advances in Enzymol.* **9,** 455 (1949).

consider the development of these modern views and to mention briefly suggestions which have been made regarding other types of linkage which might possibly be present to a minor extent.

Electrometric titration of monoribonucleotides and of ribo- and deoxyribonucleic acids by Levene and Simms[7] led directly to the general concept I for nucleic acid structure, in which the nucleoside residues were bound together by phosphodiester linkages, and eliminated from consideration ether or pyrophosphate linkages. This simple formulation of the internucleotidic linkage has now become generally accepted, and recent work has served to define the position of the linkage points in the nucleoside residues.[8] These developments will form the main theme of this chapter, but before turning to them mention may be made of the recorded evidence for other types of linkage.



$$\begin{array}{l} \text{Base—sugar—PO(OH)} \\ \qquad\text{Base—sugar—PO(OH)} \\ \qquad\qquad\text{Base—sugar—PO(OH)} \end{array}$$

I

Electrometric titration of ribonucleic acids using refined techniques[9-12] and dye-binding studies[13,14] led various workers to the conclusion that the amount of secondary phosphoryl dissociation they exhibit is greater than would be expected of a high-molecular-weight linear polydiester of type I. This led Gulland *et al.*[10,15,16] to propose structures (e.g., Ia) incorporating a number of phosphotriester linkages, with a consequent increase in the number of phosphomonoester end groups. It is perhaps significant that recent studies using nucleic acids isolated by very mild procedures have tended to minimize the proportion of secondary phosphoryl dissociation.[17-19] The general lack of agreement on this point probably stems from the use by different workers of a variety of rather ill-characterized nucleic acid preparations.

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[7] P. A. Levene and H. S. Simms, *J. Biol. Chem.* **65,** 519 (1925); **70,** 327 (1926).

[8] A. R. Todd, *Angew. Chem.* **65,** 12 (1953).

[9] F. W. Allen and J. J. Eiler, *J. Biol. Chem.* **137,** 757 (1941).

[10] W. E. Fletcher, J. M. Gulland, and D. O. Jordan, *J. Chem. Soc.* **1944,** 33.

[11] Y. Khouvine and J. Grégoire, *Bull. soc. chim. biol.* **26,** 424 (1944).

[12] H. Chantrenne, *Bull. soc. chim. Belges* **55,** 5 (1946).

[13] L. F. Cavalieri, S. E. Kerr, and A. Angelos, *J. Am. Chem. Soc.* **73,** 2567 (1951).

[14] L. F. Cavalieri, A. Angelos, and M. E. Balis, *J. Am. Chem. Soc.* **73,** 4902 (1951).

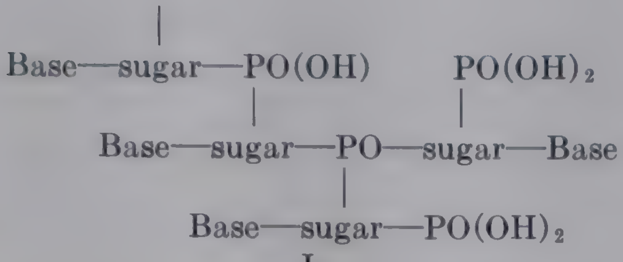
[15] J. M. Gulland and D. O. Jordan, *Nature* **161,** 561 (1948).

[16] J. M. Gulland, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 95 (1947).

[17] C. A. Zittle, *J. Biol. Chem.* **166,** 491 (1946).

[18] S. Weiner, E. L. Duggan, and F. W. Allen, *J. Biol. Chem.* **185,** 163 (1950).

[19] L. Vandendriessche, *Compt. rend. trav. lab. Carlsberg* **27,** 341 (1951).



Ia

No direct chemical evidence has been adduced in support of this type of branched structure; it would be expected to be relatively unstable both to acid and alkali and might, therefore, have been missed in chemical degradations. More recent evidence for this type of linkage derived from enzyme experiments will be considered later in connection with the general problem of chain-branching in ribonucleic acids.

Electrometric titration of deoxyribonucleic acids of high molecular weight shows only small amounts of secondary phosphoryl dissociation.[20] These acids are thus generally considered to be straight-chain polydiesters of type I, a structure which also accords with their other physical properties.[21] [Cf. *Jordan*, Chapter 13.] Nevertheless, Lee and Peacocke[22] have interpreted their titration data on the basis of a branched structure including phosphotriester linkages, a conclusion also reached from dye adsorption studies.[23]

Euler and Fonó[24] have observed the liberation of base-binding groups when deoxyribonucleic acid is treated with alkali (pH 11.5). They suggest that this represents the fission of linkages between phosphate and the enolic hydroxyl groups of purine or pyrimidine residues. A similar explanation has been suggested by Little and Butler[25] to account for their observation that during the action of deoxyribonuclease on deoxyribonucleic acids groups of pK 9–10 are liberated, in addition to secondary phosphoryl groups. It seems more likely that these represent enolic hydroxyl groups on the purine and pyrimidine residues, which are masked by hydrogen-bonding and are set free during degradation of the molecule, than that they originate in covalent internucleotidic linkages. Since the specificity and mode of action of deoxyribonuclease has not been clearly defined, it is possible, as Zamenhof and Chargaff[26] point out, that two effects may be super-

[20] J. M. Gulland, D. O. Jordan, and H. F. W. Taylor, *J. Chem. Soc.* **1947,** 1131.

[21] D. O. Jordan, *Progr. Biophys. and Biophys. Chem.* **2,** 51 (1951).

[22] W. A. Lee and A. R. Peacocke, *J. Chem. Soc.* **1951,** 3361.

[23] L. F. Cavalieri and A. Angelos, *J. Am. Chem. Soc.* **72,** 4686 (1950).

[24] H. von Euler and A. Fonó, *Arkiv. Kemi Mineral. Geol.* **25A,** No. 3 (1947).

[25] J. A. Little and G. C. Butler, *J. Biol. Chem.* **188,** 695 (1951).

[26] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **187,** 1 (1950).

imposed—an enzymic cleavage of covalent links followed by a spontaneous rupture of secondary valence bonds. Similar observations have been made by Vandendriessche[19] in a study of the degradation of yeast ribonucleic acid by ribonuclease. He, too, points out that the liberation of such titratable groups does not necessarily indicate that they were originally present in covalent linkages. Cavalieri, Kerr, and Angelos[13] report discrepancies between base content and titratable groups in the so-called "core" of ribonucleic acid, and suggest that in this material (see later), which constituted some 3–10% of the original nucleic acid, the enolic hydroxyl of uracil and guanine may be involved in internucleotidic linkages. Corresponding changes in ultraviolet absorption when this material[13,27] and intact ribonucleic acid[28] are hydrolyzed by alkali, or when deoxyribonucleic acids are degraded by deoxyribonuclease[29] or snake venom,[30] suggest, however, that the changes are more likely to originate in cleavage of secondary valence bonds, which interfere with the resonating system of purine and pyrimidine rings,[27,31] than in rupture of covalent linkages. [Cf. *Beaven, Holiday, and Johnson*, Chapter 14.]

Based on the reaction of deoxyribonucleic acids with Feulgen's reagent after very mild treatment with acid and alkali, or even simple dialysis, Stacey *et al.*[32-35] have proposed that a small number of labile phosphoryl linkages attached to C_1 of deoxyribose residues are present. This type of structure would, of course, be at variance with the generally accepted polynucleotide structure of nucleic acids, although it could be argued that a very small proportion of such links in a nucleic acid of high molecular weight might make little difference to the nitrogen–phosphorus ratio as determined by analysis.

Consideration of the various types of linkage which have been proposed in recent years leads to the conclusion that the phosphodiester linkage between the sugar residues of the individual nucleosides remains at least the major one. The others, apart possibly from the phosphotriester link, may have been advanced on reasonable grounds, but the absence of corroborative chemical evidence and, in general, their apparently very small occurrence in proportion to the others, suggests that they are of minor

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[27] B. Magasanik and E. Chargaff, *Biochim. et Biophys. Acta* **7**, 396 (1951).

[28] M. Kunitz, *J. Biol. Chem.* **164**, 563 (1946).

[29] M. Kunitz, *J. Gen. Physiol.* **33**, 349, 363 (1950).

[30] R. L. Sinsheimer and J. F. Koerner, *J. Biol. Chem.* **198**, 293 (1952).

[31] B. Commoner, *Science* **110**, 31 (1949).

[32] C. F. Li, W. G. Overend, and M. Stacey, *Nature* **163**, 538 (1949).

[33] W. G. Overend, M. Stacey, and M. Webb, *J. Chem. Soc.* **1951**, 2450.

[34] W. G. Overend, A. R. Peacocke, and M. Stacey, *J. Sci. Food Agr.* **3**, 105 (1952).

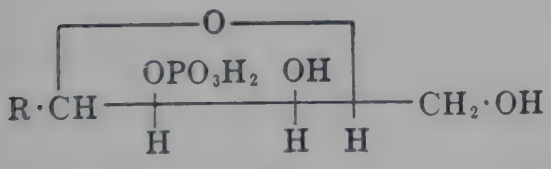
[35] W. A. Lee and A. R. Peacocke, *J. Chem. Soc.* **1952**, 130.

significance, and may even represent false interpretations of experimental findings.

II. Chemistry of the Ribonucleic Acids

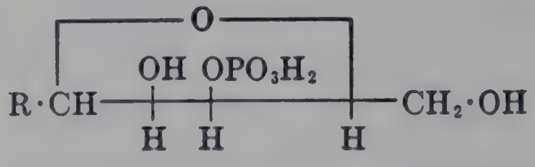
1. Mononucleotides

Until 1949 only four mononucleotides had been isolated from hydrolysates of ribonucleic acids. Levene and his co-workers[36,37] concluded from degradative studies and arguments based on analogy[2] that these were the 3′-phosphates of the four nucleosides adenosine, guanosine, cytidine, and uridine, i.e., that they could be represented by structure IIb in which R represents a purine (adenine or guanine) or pyrimidine (cytosine or uracil) residue. The fact that only these four nucleotides were obtained made it very difficult to define the position of the internucleotidic linkage. Clearly it could not be 3′,3′-, but if it were not then it seemed necessary to assume that a phosphoryl linkage at a position other than 3′- would be preferentially hydrolyzed. The 5′-position seemed unlikely on this basis since adenosine-5′-phosphate (muscle adenylic acid) had a stability towards hydrolysis comparable with that of yeast adenylic acid and Levene and Tipson[38] therefore formulated the ribonucleic acids as 2′,3′-linked polynucleotides, assuming, in the absence of any experimental evidence, that the $C_{2'}$—O—P linkage would be less stable than the $C_{3'}$—O—P linkage, and would therefore always be ruptured on hydrolysis, yielding only the nucleoside-3′-phosphates. Gulland,[3,39] on the basis of enzymic studies, did at one stage propose a 3′,5′-linked structure for ribonucleic acid, but as he found it necessary to invoke a rather improbable phosphoryl migration from $C_{5'}$ to $C_{3'}$ to account for the products of alkaline hydrolysis, he later retracted this proposal.[40]



$$\overbrace{R\cdot CH-\overset{OPO_3H_2}{\underset{H}{C}}-\overset{OH}{\underset{H}{C}}-\underset{H}{C}}^{O}-CH_2\cdot OH$$

IIa



$$\overbrace{R\cdot CH-\overset{OH}{\underset{H}{C}}-\overset{OPO_3H_2}{\underset{H}{C}}-\underset{H}{C}}^{O}-CH_2\cdot OH$$

IIb

The isolation by Carter and Cohn[41] in 1949 of two isomeric adenylic acids, termed *a* and *b*, from alkaline hydrolysates of yeast ribonucleic acid, and the subsequent demonstration that similar pairs of isomeric nucleotides

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[36] P. A. Levene and S. A. Harris, *J. Biol. Chem.* **98,** 9 (1932).

[37] P. A. Levene and S. A. Harris, *J. Biol. Chem.* **101,** 419 (1933).

[38] P. A. Levene and R. S. Tipson, *J. Biol. Chem.* **109,** 623 (1935).

[39] J. M. Gulland and E. M. Jackson, *J. Chem. Soc.* **1938,** 1492.

[40] J. M. Gulland and E. O. Walsh, *J. Chem. Soc.* **1945,** 172.

[41] C. E. Carter and W. E. Cohn, *Federation Proc.* **8,** 190 (1949), and subsequent papers.

derived from each of the other three nucleosides were also produced[42-4 put a new complexion on the polynucletide problem. The *a* and *b* nucleotides were shown[46] to be the 2′- and 3′-phosphates of the corresponding nucleosides (i.e., IIa and b), although not necessarily respectively, and much subsequent work has confirmed this conclusion. The acid-catalyzed interconversion of the isomeric *a* and *b* nucleotides,[43,46] which will be discussed later, rendered invalid the conclusion of Levene and Harris that their isolated nucleotides were 3′-phosphates, since their degradative method could not really distinguish between the 2′- and 3′-isomers. Very recent studies[47-50] have provided strong evidence for the view that the *a* nucleotides are the 2′-phosphates (IIa), and the *b* nucleotides the 3′-phosphates (IIb), of the respective nucleosides. While additional confirmation[50a] of these findings would no doubt be desirable, it is reasonable to accept them for the purposes of our discussion. On their validity rests any final conclusion regarding the absolute orientation of the internucleotidic linkages in intact ribonucleic acids.

The salient points in the chemistry of the nucleoside-2′- and -3′-phosphates [cf. *Baddiley*, Chapter 4] may be briefly restated. Their interconversion in acid solution to an equilibrium mixture[43,46] has been shown[46] by analogy with the glycerol-α- and -β-phosphates[51-53] to depend on migration of the phosphoryl group via an intermediate cyclic 2′,3′-phosphate (III). The intermediate cyclic 2′,3′-phosphates have been synthesized[54] and shown to possess the predicted properties, i.e., they are unstable and

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[42] W. E. Cohn, *J. Am. Chem. Soc.* **72,** 1471 (1950).

[43] W. E. Cohn, *J. Am. Chem. Soc.* **72,** 2811 (1950).

[44] H. S. Loring, N. G. Luthy, H. W. Bortner, and L. W. Levy, *J. Am. Chem. Soc.* **72,** 2811 (1950).

[45] W. E. Cohn, *J. Cellular Comp. Physiol.*, **38,** Suppl. 1, 21 (1951).

[46] D. M. Brown and A. R. Todd, *J. Chem. Soc.* **1952,** 44.

[47] H. S. Loring, M. L. Hammell, L. W. Levy, and H. W. Bortner, *J. Biol. Chem.* **196,** 821 (1952).

[48] L. F. Cavalieri, *J. Am. Chem. Soc.* **74,** 5804 (1952); **75,** 5268 (1953).

[49] J. J. Fox, L. F. Cavalieri, and N. Chang, *J. Am. Chem. Soc.* **75,** 4315 (1953).

[50] J. X. Khym, D. G. Doherty, E. Volkin, and W. E. Cohn, *J. Am. Chem. Soc.* **75,** 1262 (1953).

[50a] This has recently been given in the case of the isomeric adenylic acids by X-ray analysis (D. M. Brown, G. D. Fasman, D. I. Magrath, A. R. Todd, W. Cochran, and M. M. Woolfson, *Nature* **172,** 1184, 1953), by chemical synthesis (*loc. cit.*; D. M. Brown, G. D. Fasman, D. I. Magrath, and A. R. Todd, *J. Chem. Soc.* **1954,** 1448), and by degradation (J. X. Khym and W. E. Cohn, *J. Am. Chem. Soc.* **76,** 1818, 1954).

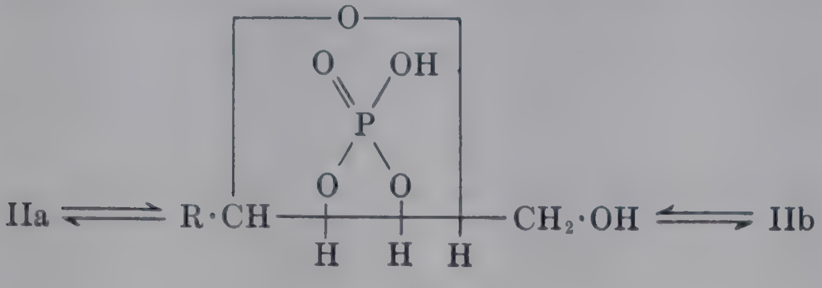
[51] M. C. Bailly, *Compt. rend.* **206,** 1902 (1938); **208,** 443 (1939).

[52] P. E. Verkade, J. C. Stoppelenburg, and W. D. Cohen, *Rec. trav. chim.* **59,** 886 (1940).

[53] E. Chargaff, *J. Biol. Chem.* **145,** 455 (1942).

[54] D. M. Brown, D. I. Magrath, and A. R. Todd, *J. Chem. Soc.* **1952,** 2708.

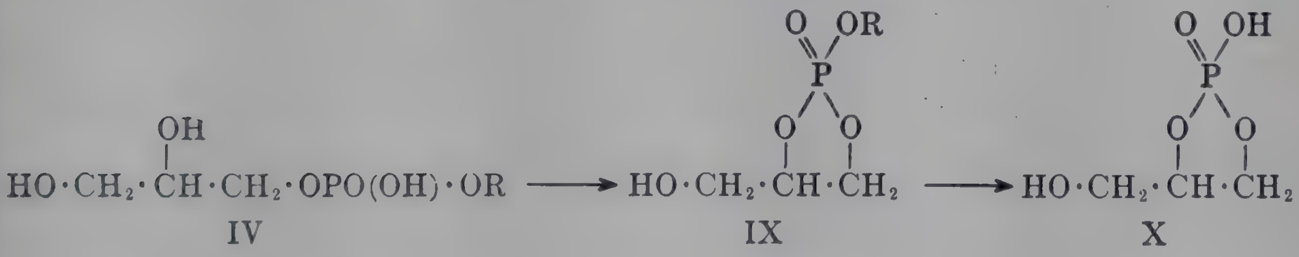
are readily hydrolyzed to a mixture of the nucleoside-2′- and nucleoside-3′-phosphates. The free isomeric mononucleotides are, like the glycerol monophosphates, stable without interconversion in alkaline solution.



III

2. Esters of the Mononucleotides

Bailly and Gaumé[55] showed that whereas glycerol-α-phosphate is stable to alkali, glycerol-α methyl hydrogen phosphate (IV; R = Me) is readily hydrolyzed under alkaline conditions to methanol and a mixture of glycerol-α- and -β-phosphate; no methyl phosphate is produced. Similar observations by Baer and Kates[56] show that the choline phosphate (IV; R = choline residue) is hydrolyzed to choline and glycerol-α- and -β-phosphate, while alkaline degradation of the lecithins also results in phosphoryl migration.[57] 2-Hydroxyethyl dimethyl phosphate (V) under the action of dilute alkali yields 2-hydroxyethyl phosphate (VI) with loss of methanol.[58] Mild acid treatment also effects these degradations. It is noteworthy, however, that 2-methoxyethyl dimethyl phosphate (VII) forms 2-methoxyethyl methyl hydrogen phosphate (VIII) with alkali, and this is now stable to further hydrolysis, a result which is in accord with the generally recognized stability of dialkyl esters of phosphoric acid towards alkaline reagents.[59-61]



$$\underset{\text{V}}{HO \cdot CH_2 \cdot CH_2 \cdot OPO(OMe)_2} \longrightarrow \underset{\text{VI}}{HO \cdot CH_2 \cdot CH_2 \cdot OPO_3H_2}$$

$$\underset{\text{VII}}{MeO \cdot CH_2 \cdot CH_2 \cdot OPO(OMe)_2} \longrightarrow \underset{\text{VIII}}{MeO \cdot CH_2 \cdot CH_2 \cdot OPO(OH) \cdot OMe}$$

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[55] O. Bailly and J. Gaumé, *Bull. soc. chim.* **2,** 354 (1935).

[56] E. Baer and M. Kates, *J. Biol. Chem.* **175,** 79 (1948).

[57] E. Baer and M. Kates, *J. Biol. Chem.* **185,** 615 (1950).

[58] O. Bailly and J. Gaumé, *Bull. soc. chim.* **3,** 1396 (1936).

[59] J. Cavalier, *Compt. rend.* **127,** 114 (1898).

The prerequisite for alkali-lability in dialkyl phosphates is thus the presence of hydroxyl function in proximity to the phosphoryl group.

It is well known that triesters of phosphoric acid are sensitive both to alkali and to acid.[60,61] Brown and Todd[62] envisaged a mechanism for the hydrolysis of the hydroxylated dialkyl phosphates in which a cyclic intermediate is involved. Thus, in the alkaline hydrolysis of glycerol-α methyl phosphate (IV; R = Me) an intermediate *e.g.* IX (R = Me) is produced and immediately cleaved to methanol and the cyclic phosphate X which then gives rise to glycerol-α- and -β-phosphate. No other major products are to be expected, since it is evident that although the three ester linkages are of comparable reactivity, yet only by fission of the bond retaining the singly-linked substituent can degradation of the molecule occur. Other structures for the intermediate IX have been proposed.[56,62] Brown and Todd[63] point out that the classical neutral triester intermediate of type IX was originally advanced to simplify discussion, and suggest that the true mechanism of processes of type IV → X depends probably on an acid- or base-catalyzed attack by the vicinal hydroxyl group on the —P=O bond with simultaneous elimination of the R group as an alkoxy anion, possibly with the intervention of a pentacovalent transition complex.[63a]

Fonó,[64] as early as 1947, noted the importance of the neighboring hydroxyl groups in causing lability of diesters of phosphoric acid containing a glycerol or ethylene glycol residue, and postulated the existence of a cyclic triester in their hydrolysis. He put forward the view that the alkali-lability of ribonucleic acid, in contrast to the alkali-stability of deoxyribonucleic acid, may depend on the extra hydroxyl group present at C_2 in the sugar residues of the former, i.e., that ribonucleic acids are analogous to glycerol alkyl phosphates in their hydrolytic behavior. Although evidence was not available at the time to permit detailed conclusions to be drawn, subsequent work has clearly established the validity of Fonó's basic idea.

Brown and Todd,[46,62] during their synthesis of adenylic acids *a* and *b*, isolated two substances shown to be adenosine-2′ benzyl phosphate and adenosine-3′ benzyl phosphate (XI and XII; R′ = $CH_2C_6H_5$; R = adenine residue). These substances were found to be readily hydrolyzed in both

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[60] R. H. A. Plimmer and W. J. N. Burch, *J. Chem. Soc.* **1929,** 279.

[61] G. M. Kosolapoff, "Organophosphorus Compounds," p. 232. John Wiley & Sons, New York, 1950.

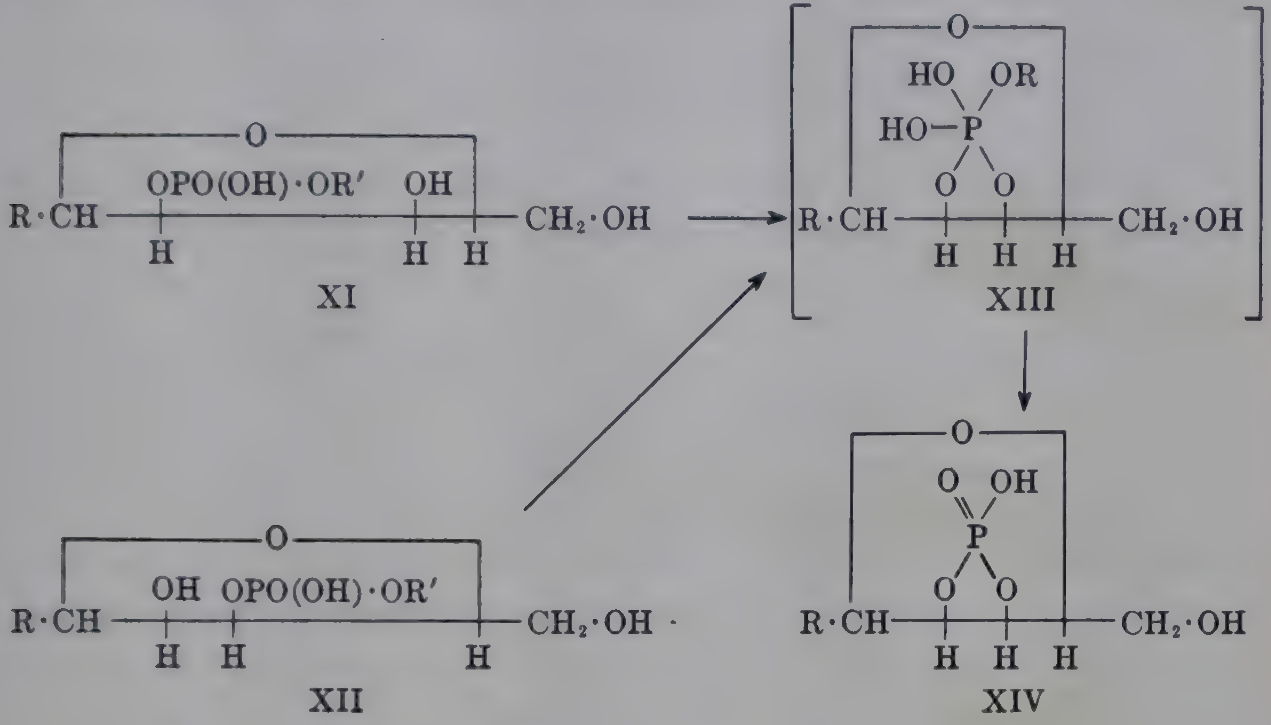
[62] D. M. Brown and A. R. Todd, *J. Chem. Soc.* **1952,** 52.

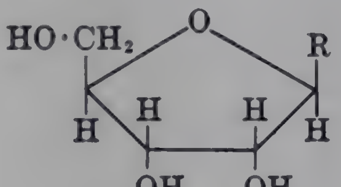
[63] D. M. Brown and A. R. Todd, *J. Chem. Soc.* **1953,** 2040.

[63a] This view of the mechanism finds confirmation in the experiments of D. Lipkin, P. T. Talbert and M. Cohn (*J. Am. Chem. Soc.* **76,** 2871, 1954) who showed that alkaline hydrolysis of yeast ribonucleic acid in H_2O^{18} yields mononucleotides containing only one atom of O^{18} per atom of phosphorus

[64] A. Fonó, *Arkiv. Kemi Mineral. Geol.* **24A,** No. 33, 14, 15 (1947).

acidic and alkaline media with simultaneous phosphoryl migration yielding benzyl alcohol and a mixture of adenosine-2′- and -3′-phosphate; no other products were observed. Clearly these degradations are entirely analogous to those recorded in the glycerol phosphate series, and proceed by way of a cyclic intermediate (e.g., XIII); this then yields the cyclic phosphate XIV (R = adenine residue), which subsequently hydrolyzes to adenosine-2′- and -3′-phosphate. The properties of the synthetic nucleoside-2′,3′-phosphates[54] (XIV) are in every way consistent with the hypothesis that they participate in the hydrolytic breakdown of nucleotide esters. Comparable observations have since been made on benzyl, methyl, and ethyl esters of the cytidylic and uridylic acids.[54,63]





XV

It was also noted that adenosine-5′ benzyl phosphate[65] was stable under conditions which led to the hydrolysis of the 2′- and 3′-nucleotide esters. Nor was adenosine-5′-phosphate converted under acidic conditions into the 2′- or 3′-nucleotide.

Consideration of the stereochemistry of the natural ribonucleosides (XV; R = purine or pyrimidine residue) shows that the hydroxyl groups at C_2 and C_3 of the sugar residue bear a *cis*-relationship to each other, thus per-

[65] J. Baddiley and A. R. Todd, *J. Chem. Soc.* **1947**, 648.

mitting ready formation of cyclic 2′,3′-phosphoryl intermediates in the interconversions of the 2′- and 3′-nucleotides, whereas the *trans*-relationship between the hydroxyl groups at C_3 and C_5 prevents the formation of cyclic phosphoryl and other[66] derivatives at these positions. As a corollary, it is evident from the above that the course of hydrolysis of analogous derivatives would be expected to be different if sugar residues (e.g., xylofuranose), in which the stereochemical relationships of the hydroxyl functions were altered, were substituted for ribofuranose in the nucleosides. Ribose and deoxyribose in their furanose forms are uniquely suited for incorporation in the nucleic acids.

The above observations on the reactions of simple esters of the mononucleotides can be applied directly to the elucidation of ribonucleic acid structure.

3. General Structure of Polyribonucleotides Based on Chemical Degradation

It was early shown that when ribonucleic acids are treated with mild alkaline reagents under a variety of conditions they are rapidly converted to a mixture of their component mononucleotides.[67-72] [Cf. Chapters 5 and 11.] Claims to the isolation of larger fragments[73,74] have generally been relinquished or refuted by other workers on the general grounds that the products described were separable mixtures of mononucleotides[75,76] (compare, however, Smith and Allen[77]). In the same way, mild acid hydrolysis also yields mononucleotides, although further degradation of the purine nucleotides complicates the picture. The early observation that the final products of alkaline hydrolysis are mononucleotides has been confirmed by recent studies using chromatographic[45,78-80] and electrophoretic[81] separation, and physical methods for identifying the products. Recovery of the mononucleotides as mixtures of the 2′- and 3′-isomers (generally in the ratio 40:60) from alkaline hydrolysates of ribonucleic acids, in amounts approaching the theoretical value, have been reported.[45,82]

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[66] D. M. Brown, L. J. Haynes, and A. R. Todd, *J. Chem. Soc.* **1950,** 3299.

[67] H. Steudel and E. Peiser, *Z. physiol. Chem.* **120,** 292 (1922).

[68] P. A. Levene, *J. Biol. Chem.* **40,** 415 (1919); **55,** 9 (1923).

[69] W. Jones and M. E. Perkins, *J. Biol. Chem.* **62,** 557 (1925).

[70] H. O. Calvery, *J. Biol. Chem.* **72,** 27 (1927).

[71] G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.* **161,** 83 (1945).

[72] H. S. Loring, P. M. Roll, and J. G. Pierce, *J. Biol. Chem.* **174,** 729 (1948).

[73] W. Jones and H. C. Germann, *J. Biol. Chem.* **25,** 93 (1916); W. Jones and B. E. Read, *ibid.* **29,** 111; **31,** 39 (1917).

[74] S. J. Thannhauser and G. Dorfmüller, *Z. physiol. Chem.* **95,** 259 (1915).

[75] P. A. Levene, *J. Biol. Chem.* **33,** 229 (1919).

[76] W. Jones, "Nucleic Acids," p. 36. Longmans, Green and Co., London, 1920.

[77] K. C. Smith and F. W. Allen, *J. Am. Chem. Soc.* **75,** 2131 (1953).

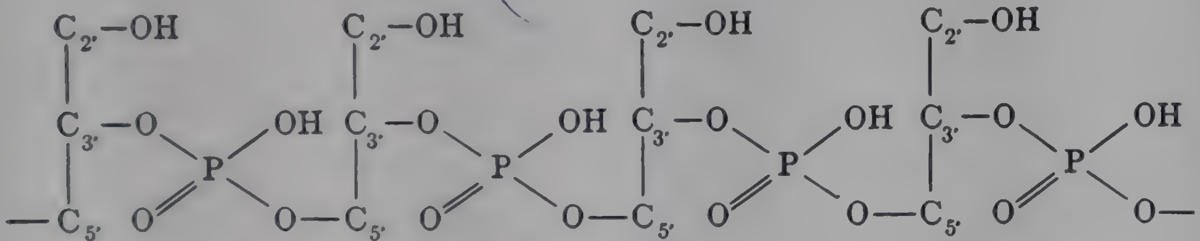
[78] E. Chargaff, B. Magasanik, E. Vischer, C. Green, R. Doniger, and D. F. Elson, *J. Biol. Chem.* **186,** 51 (1950).

[79] R. Markham and J. D. Smith, *Biochem. J.* **49,** 401 (1951).

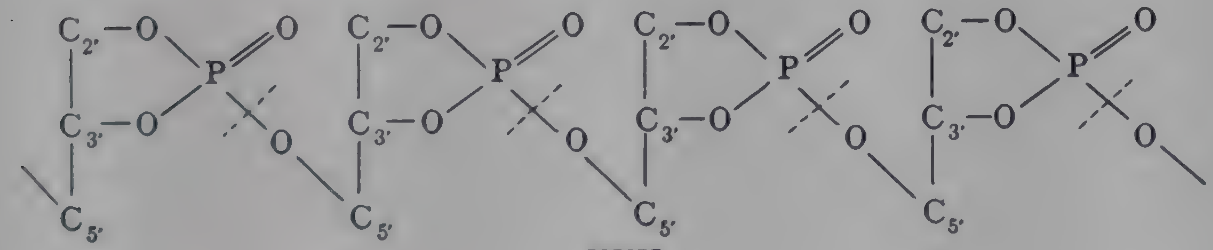
[80] J. Montreuil and P. Boulanger, *Bull. soc. chim. biol.* **33,** 784, 791 (1951).

[81] J. N. Davidson and R. M. S. Smellie, *Biochem. J.* **52,** 594 (1952).

Brown and Todd[62] have discussed these observations on the basis of their findings with the esters of the mononucleotides (see above), and have developed from them general structures for the ribonucleic acids. If the linear polynucleotide sequence XVI is considered, in which the expression $C_{2'}$—$C_{3'}$—$C_{5'}$ is used as an abbreviation for individual nucleoside residues, alkaline degradation will proceed through a cyclic intermediate (formulated for simplicity as XVII) yielding cyclic nucleoside-2′,3′-phosphates by *exclusive* fission of the $C_{5'}$—O—P bond, in strict conformity with the behavior of the fundamentally analogous simple nucleotide esters. The cyclic phosphates then yield, by further hydrolysis, the mixture of nucleoside-2′-phosphates and nucleoside-3′-phosphates which is normally isolated. This postulate that cyclic nucleoside-2′,3′-phosphates should be produced during alkaline hydrolysis of ribonucleic acids has since been substantiated by their demonstration[83] in barium carbonate (*ca.* pH 9 at 100°) and dilute ammonia hydrolysates. Kinetic studies, too, give results consistent with the proposed hydrolytic mechanism.[84] The earlier failure to recognize cyclic phosphates among hydrolytic products was doubtless due to their labile character.



XVI



XVII

It should be noted that no such mechanism of hydrolysis can occur in the case of deoxyribonucleic acids where the absence of a hydroxyl at $C_{2'}$ in the deoxyribofuranose residues prevents the essential cyclization which

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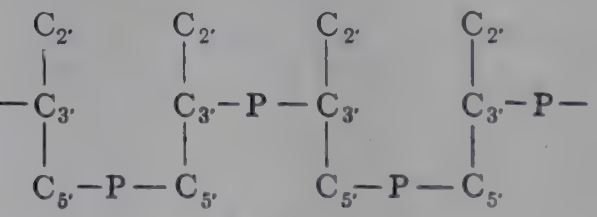
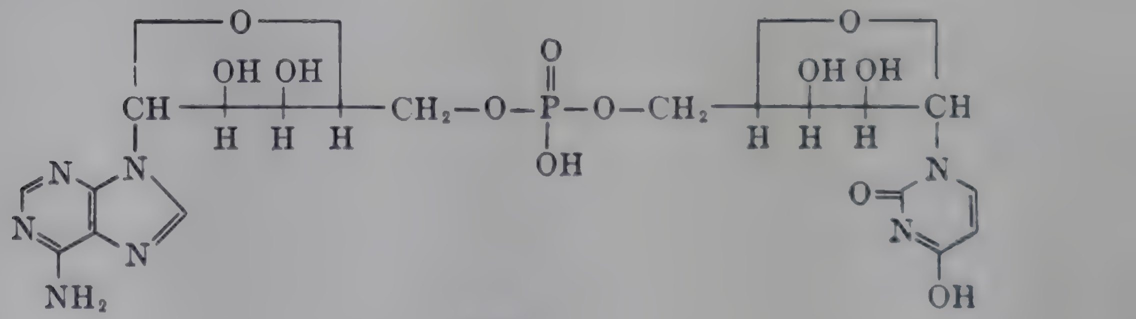
[82] E. Volkin and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1516 (1951).
[83] R. Markham and J. D. Smith, *Biochem. J.* **52,** 552 (1952).
[84] J. E. Bacher and W. Kauzmann, *J. Am. Chem. Soc.* **74,** 3779 (1952).

is a prerequisite for alkali-lability. It is for this reason that they are no degraded to small molecules by alkali,[67,71] and their stability in this respec is in accord with the generally accepted resistance of dialkyl phosphates t alkaline hydrolysis.[61,62] Acid hydrolysis of deoxyribonucleic acids clearl depends on other factors and will be discussed later.

Arguments based on complete hydrolysis to mononucleotides do not, o themselves, permit rigid conclusions to be drawn regarding the precis position of the internucleotidic linkages in ribonucleic acids, but they certainly limit the number of possible structures. In addition to structur XVI, in which the linkage is shown joining $C_{3'}$ in one nucleoside residu to $C_{5'}$ in the next, a comparable structure in which $C_{2'}$ is linked to $C_{5'}$ ca be drawn, which would be indistinguishable from XVI by alkaline hydrolysis. Such structures as XVIII, which involve $C_{5'}$—$C_{5'}$ linkages can, however, be dismissed, since they should yield dinucleotides stable to furthe hydrolysis. This conclusion that $C_{5'}$—$C_{5'}$ linkages are incompatible wit hydrolysis to mononucleotides is implicit in the stability of adenosine-5 benzyl phosphate towards alkali and acid, and is confirmed by the stabilit of synthetic dinucleoside-5′,5′-phosphates. Diuridine-5′,5′-diphosphate[8 (originally synthesized by Gulland and Smith, and believed erroneously t be the 2′,2′-isomer), and adenosine-5′ uridine-5′ phosphate[86] (XIX) ar both stable to alkali under conditions which bring about complete hydrolysis of ribonucleic acid to mononucleotides; this stability is due to the absence of the vicinal hydroxyl group necessary for cyclization and henc easy fission of the phosphoryl group in these substances.



XVIII



XIX

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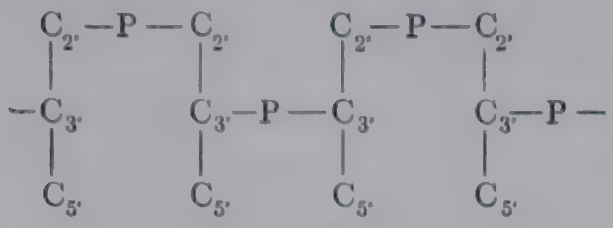
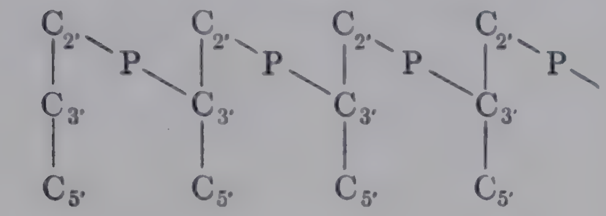
[85] J. M. Gulland and H. Smith, *J. Chem. Soc.* **1948,** 1532.
[86] D. T. Elmore and A. R. Todd, *J. Chem. Soc.* **1952,** 3681.



XX



XXI

Two other structural types (XX and XXI, or structures containing the features of both) would also yield mononucleotides by the hydrolytic mechanism under discussion. In each case, however, hydrolysis would necessarily have to proceed stepwise by removal of mononucleotide units from that end of the polynucleotide chain bearing a free hydroxyl group at $C_{2'}$ or $C_{3'}$. Structures of type XVI, embodying a 3′,5′-linkage, could in theory be degraded by simultaneous attack at many points in the chain. Relevant chemical evidence, although scanty, is in favor of simultaneous rather than stepwise attack.[87] (Compare, however, Magasanik and Chargaff.[27]) Merrifield and Woolley[87] showed that controlled acid hydrolysis of yeast ribonucleic acid yielded a variety of small oligonucleotides, some of which they separated by ion-exchange chromatography and were able to characterize. Production of molecules of this type shows that structural features in type XVI, in which 3′,5′(or 2′,5′)-linkages are present, must exist in ribonucleic acids as distinct from such structures as XX and XXI, where $C_{5'}$ is not involved in the internucleotidic linkage and where hydrolysis, which requires a free hydroxyl at $C_{2'}$ or $C_{3'}$, can only occur at a terminal linkage; in other words $C_{5'}$ must be involved as one of the internucleotidic linkage points. The isolation of nucleoside-5′-phosphates after enzymic hydrolysis of ribonucleic acids to be discussed later also indicates that $C_{5'}$ is one of the major linkage points.

The above considerations led Brown and Todd[62] to postulate the general structure XVI, involving a recurring 3′,5′-phosphodiester linkage for the polynucleotide sequence in ribonucleic acids, as the one which accounts most satisfactorily for the chemical evidence. They pointed out, however, that a 2′,5′-linkage would be equally admissible on the known facts of hydrolysis. Preference for the 3′,5′-structure was expressed mainly on the ground of analogy with deoxyribonucleic acids where a 2′,5′-linkage is structurally impossible; justification for this preference has since come from further studies using enzymes. Evidence from chemical hydrolysis to mononucleotides does not permit any answer to the question whether ribonuclei acids are straight-chain or branched-chain polynucleotides. It does, however, clearly define the types of branching which can be considered. Give that the polynucleotide chain in each branch follows the standard 3′,5′ linkage pattern, then two types of branching can be envisaged: (a) branching on phosphorus, i.e., through alkali-labile phosphotriester groupings and (b) branching at $C_{2'}$ in one of the nucleoside residues in the main chain attachment being through the usual phosphodiester linkage to $C_{3'}$ (or $C_{2'}$ in the first nucleoside residue of the branch; linkage to $C_{5'}$ would give a alkali-stable, and therefore inadmissible, structure. The question of chain branching in ribonucleic acids will be discussed later; it is mentioned a this point because the evidence of chemical hydrolysis clearly defines th only types of branching which need be considered.

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[87] R. B. Merrifield and D. W. Woolley, *J. Biol. Chem.* **197,** 521 (1952).

4. Evidence for Nucleoside-5′-Linkages

Although evidence has gradually accumulated, mainly as a result of enzymic studies, that 5′-phosphoester linkages occur in nucleic acids, the complete failure to isolate nucleoside-5′-phosphates from chemical hydrolysates for long prevented the general acceptance of their presence. Following early studies by Takahashi[88] and Klein and Rossi,[89] Gulland and Jackson[39] examined the action of Russell's viper venom. This venom had in addition to 5′-nucleotidase and phosphodiesterase, a weak nonspecific monoesterase activity; however, they showed that when it acted on yeast ribonucleic acid it liberated 25% of the bound phosphorus as inorganic phosphate, and this was increased to 75% by the addition of bone phosphomonoesterase. These early indications of 5′-phosphoester linkages have been confirmed in recent years by the work of Cohn and his co-workers. Thus Cohn and Volkin[90] treated ribonucleic acid with ribonuclease, followed by intestinal phosphatase in the presence of arsenate, to inhibit phosphomonoesterase.[91] Analysis of the hydrolysate by ion-exchange chromatography yielded, in addition to unidentified products, the 5′-phosphates of adenosine, guanosine, uridine, and cytidine. These were characterized by comparison with the synthetic nucleoside-5′-phosphates prepared by Michelson and Todd.[92] Using venom diesterase virtually free from monoesterase (Hurst, Little, and Butler[93]), Cohn and Volkin[94] obtained a yield of

[88] H. Takahashi, *J. Biochem. Japan* **16,** 463 (1932).
[89] W. Klein and A. Rossi, *Z. physiol. Chem.* **231,** 104 (1935).
[90] W. E. Cohn and E. Volkin, *Nature* **167,** 483 (1951).
[91] *cf.* W. Klein, *Z. physiol. Chem.* **218,** 164 (1933).
[92] A. M. Michelson and A. R. Todd, *J. Chem. Soc.* **1949,** 2476.
[93] R. O. Hurst, J. A. Little, and G. C. Butler, *J. Biol. Chem.* **188,** 705 (1951).
[94] W. E. Cohn and E. Volkin, *Arch. Biochem. and Biophys.* **35,** 465 (1952).

more than 60% of nucleoside-5′-phosphates from the ribonucleic acids of yeast and calf liver, in addition to some free nucleosides and nucleoside diphosphates. These latter substances, which are discussed later in connection with chain-branching, have one of their phosphate groups at the 5′-position of the nucleoside residue. Thus there is good evidence that the $C_{5'}$-position is of major importance as an internucleotidic linkage point. Further strong confirmatory evidence is to be found in the chemistry of the oligonucleotides found in ribonuclease digests of ribonucleic acids. The presence of 5′-phosphoester linkages in ribonucleic acids and the absence of nucleoside-5′-phosphates in chemical hydrolysates find an adequate explanation in the theory of hydrolysis discussed above.[62]

5. Chemistry of Ribonuclease Action

As clearly indicated, alkaline hydrolysis degrades ribonucleic acid to mononucleotides without yielding larger fragments (oligonucleotides) containing more than one nucleotide unit. Such larger fragments are of importance in the study both of nucleotide sequence and of the detail of the internucleotidic linkage. They can be obtained by the action of certain enzymes, among which the most widely used is pancreatic ribonuclease. This enzyme was obtained in crystalline form by Kunitz[95] in 1940, and its use in recent years has shed light on a number of important details of ribonucleic acid structure.

Allen and Eiler[9] found that 0.25 equivalents of secondary phosphoryl groups per atom of phosphorus were liberated during ribonuclease action, and higher values (0.4–0.5) have since been reported;[82,96] no inorganic phosphate was liberated. The enzyme, indeed, seemed to be a specific type of diesterase and as such it gave both dialyzable and nondialyzable fission products when allowed to act on ribonucleic acids. Weiner, Duggan, and Allen[18] found by titration that the ratios of monoesterified to diesterified phosphate in the intact nucleic acid, and in the dialyzable and the nondialyzable fractions of the digests, were 1:10, 1:2, and 1:3, indicating that considerable breakdown had occurred even in the nondialyzable fragments. Analysis of the fractions obtained[97] showed that the smaller (dialyzable) fragments were rich in pyrimidine nucleotide derivatives, while the larger (nondialyzable) showed a high purine–pyrimidine ratio. These observations led to the suggestion[97,98] that the enzyme acted at pyrimidine nucleotide sites in the molecule. Evidence pointing in the same direction came

[95] M. Kunitz, *J. Gen. Physiol.* **24,** 15 (1940).

[96] E. Volkin and W. E. Cohn, *J. Biol. Chem.* **205,** 767 (1953).

[97] J. E. Bacher and F. W. Allen, *J. Biol. Chem.* **183,** 633 (1950).

[98] See also G. Schmidt, R. Cubiles, B. H. Swartz, and S. J. Thannhauser, *J. Biol. Chem.* **170,** 759 (1947); G. Schmidt, R. Cubiles, and S. J. Thannhauser, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 161 (1947).

from the demonstration[27,99,100] that in addition to larger fragments, sub substantial amounts of pyrimidine mononucleotides were formed by ribonuclease digestion without the concomitant production of purine mononucleotides. A single claim to have isolated purine mononucleotides from ribonuclease digests[101] has not been confirmed by later work. Further evidence for a specificity of ribonuclease towards pyrimidine nucleotide residues came from the work of Schmidt, Thannhauser, and their co-workers It was found[100,102] that after exhaustive digestion of ribonucleic acid with ribonuclease and subsequent treatment with prostatic phosphomonoesterase, inorganic phosphate was produced in an amount corresponding to at least 93 % of the pyrimidine nucleotide phosphorus content of the origina nucleic acid. The remaining organically bound phosphorus was present entirely, or almost entirely, as purine nucleotide phosphorus.[103] The conclusion was drawn that in ribonuclease digests the pyrimidine nucleotide residues were present either as mononucleotides or as terminal residues carrying a monoesterified phosphoryl group in oligonucleotides consisting otherwise solely of purine nucleotide residues. The same workers added materially to this conclusion by periodate oxidation studies. Ribonucleic acid and the products of its digestion with ribonuclease were stable to periodic acid, but after treating the digest with phosphomonesterase, a large periodate uptake was observed which was equivalent to the amount of inorganic phosphate liberated, and hence corresponded approximately to the pyrimidine nucleotide content of the original nucleic acid. They also showed that the larger, phosphorus-containing, fragments in the phosphomonoesterase-treated digests, i.e., oligonucleotides containing a terminal pyrimidine residue, were also susceptible to periodate oxidation. Since oxidation of nucleotides by periodate depends on the presence of unsubstituted hydroxyl groups at both $C_{2'}$ and $C_{3'}$,[104] they concluded that the terminal (pyrimidine) residue in these oligonucleotides was linked to the rest of the molecule through a position other than $C_{2'}$ or $C_{3'}$. Claims that ribonucleic acids themselves, and ribonuclease digests *before* phosphomonoesterase treatment, reduce periodate[13, 105] and lead tetraacetate[106] have been denied in more recent publications.[96,102] Periodate uptake *after* phospho-

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[99] C. E. Carter and W. E. Cohn, *J. Am. Chem. Soc.* **72,** 2604 (1950).

[100] G. Schmidt, R. Cubiles, and S. J. Thannhauser, *J. Cellular Comp. Physiol.* **38,** Suppl. 1, 61 (1951).

[101] H. S. Loring and F. H. Carpenter, *J. Biol. Chem.* **150,** 381 (1943).

[102] G. Schmidt, R. Cubiles, N. Zöllner, L. Hecht, N. Strickler, K. Seraidarian, M. Seraidarian, and S. J. Thannhauser, *J. Biol. Chem.* **192,** 715 (1951).

[103] *cf.* W. Jones, *J. Biol. Chem.* **24,** iii (1916).

[104] B. Lythgoe and A. R. Todd, *J. Chem. Soc.* **1944,** 592.

[105] F. W. Allen, *Federation Proc.* **10,** 155 (1951).

[106] R. A. Becher and F. W. Allen, *J. Biol. Chem.* **195,** 429 (1952).

monoesterase treatment has, on the other hand, been confirmed in several laboratories.[14,96] Brown and Todd[62] pointed out that an uptake of periodate after ribonuclease action but without phosphomonoesterase treatment would only be possible if $C_{2'}$—O—P linkages were split. The picture of ribonuclease action which emerges from all these observations is that the enzyme cleaves $C_{5'}$—O—P linkages in the postulated structure XVI giving, in addition to pyrimidine-2′- or -3′-mononucleotides, fragments containing terminal pyrimidine nucleotide residues, bearing monoesterified phosphoryl groups at $C_{2'}$ or $C_{3'}$; enzymic removal of these phosphomonoester groups then yields periodate-oxidizable pyrimidine nucleoside residues linked through $C_{5'}$ to the rest of the (purine) polynucleotide chain.

When ribonucleic acid is treated for short periods with ribonuclease, two substances are produced[107] which are converted, respectively, to uridylic and cytidylic acids by further action of the same enzyme. These two substances have been shown to be identical[62,108] with the cyclic phosphates, uridine-2′,3′-phosphate and cytidine-2′,3′-phosphate, synthesized by Brown, Magrath, and Todd.[54] It is clear that these cyclic phosphates are the precursors of the pyrimidine mononucleotides found in ribonuclease digests and that they must have arisen by a mechanism akin to that postulated for the chemical hydrolysis of the nucleic acids.[62] This was confirmed by Brown, Dekker, and Todd[109] in a further investigation of the action of ribonuclease on the cyclic phosphates. Volkin and Cohn[110] had shown that the pyrimidine nucleotide fraction in ribonuclease digests consists solely of uridylic acid *b* and cytidylic acid *b* (i.e., the 3′-phosphates), and Brown, Dekker, and Todd provided an adequate explanation of this fact by showing that ribonuclease effects a unidirectional cleavage of the cyclic 2′,3′-phosphates of uridine and cytidine yielding exclusively the *b*(3′)-isomers; ribonuclease, however, had no action on the cyclic 2′,3′-phosphates of adenosine and guanosine, a fact which supports the specificity of ribonuclease for pyrimidine nucleotide derivatives. In the course of this investigation it was shown that the phosphoryl group in cytidylic acid *b* occupied the same position (probably 3′) as in uridylic acid *b* by converting the former to the latter by deamination under alkaline conditions which precluded phosphoryl migration. In both the cyclic pyrimidine nucleotides, then, it was clear that ribonuclease cleaved the $C_{2'}$—O—P linkage. It is of some interest to note in connection with the demonstration that cyclic phosphates are intermediates in ribonuclease digestion that in 1946 Schramm, Bergold, and Flammersfeld[111] had suggested the presence of

[107] R. Markham and J. D. Smith, *Research* **4,** 344 (1951).

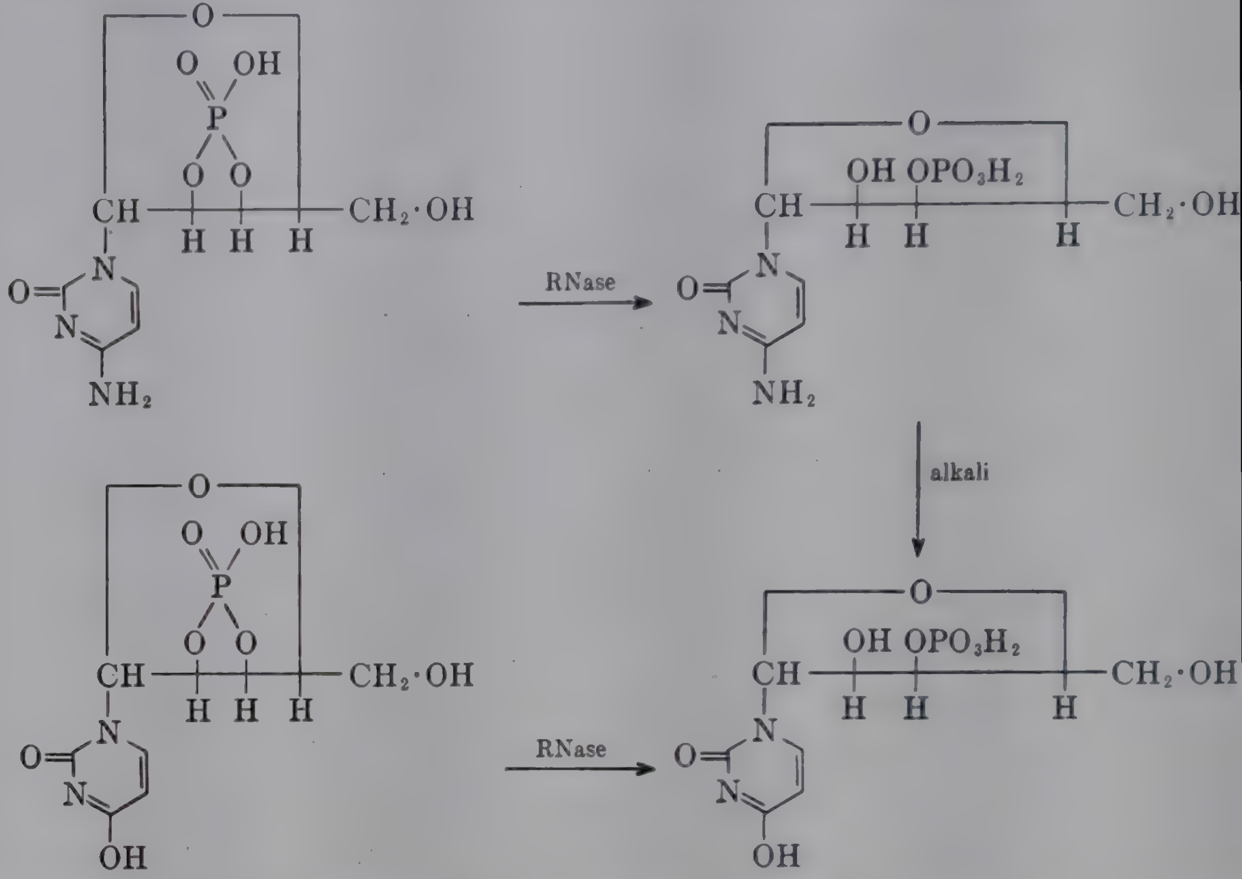
[108] R. Markham and J. D. Smith, *Nature* **168,** 406 (1951).

[109] D. M. Brown, C. A. Dekker, and A. R. Todd, *J. Chem. Soc.* **1952,** 2715.

[110] E. Volkin and W. E. Cohn, *Federation Proc.* **11,** 303 (1952).

[111] G. Schramm, G. Bergold, and H. Flammersfeld, *Z. Naturforsch.* **1,** 328 (1946).

"inner esterified" nucleotides in ribonuclease digests. The evidence for th suggestion, however, rested on the disparity between the number of acid functions liberated by the enzyme in relation to the conclusion, now know to be erroneous,[112] that the products were all mononucleotidic.



Markham and Smith[113] have studied the oligonucleotide fragments i ribonuclease digests by paper chromatographic and paper electrophoreti techniques, thereby separating a variety of di- and trinucleotides. The found that during the rapid initial action of the enzyme substances wer produced which were then transformed more slowly into the oligonucleo tides without change in the gross analytical composition (base–phosphoru ratio). They suggested, on the basis of the hydrolytic mechanism advance by Brown and Todd,[62] that these initially produced substances carried cyclic 2′,3′-phosphoryl group on the terminal (pyrimidine) nucleosid residue; further action of the enzyme on these cyclic groups then yielde the true oligonucleotides. Brown, Dekker, and Todd[109] pointed out tha this latter process, being analogous to that studied in their work on the ac tion of ribonuclease on cyclic phosphates of uridine and cytidine, shoul yield oligonucleotides bearing a terminal *b*(3′)-nucleotide residue, anc hence that on alkaline hydrolysis pyrimidine *b* nucleotides would be pro duced from them. In agreement with this view, Volkin and Cohn[96] founc that digestion of ribonucleic acid with ribonuclease, followed by treatment with alkali, yielded only the *b* isomers of the pyrimidine nucleotides, together with a mixture of the *a* and *b* isomers of the purine nucleotides. The processes involved are shown in the annexed formulas in which Py = uracil or cytosine residue and R = remainder of polynucleotide chain; where R = H the final products of ribonuclease action are the pyrimidine *b* mononucleotides which are unaffected by treatment with alkali.

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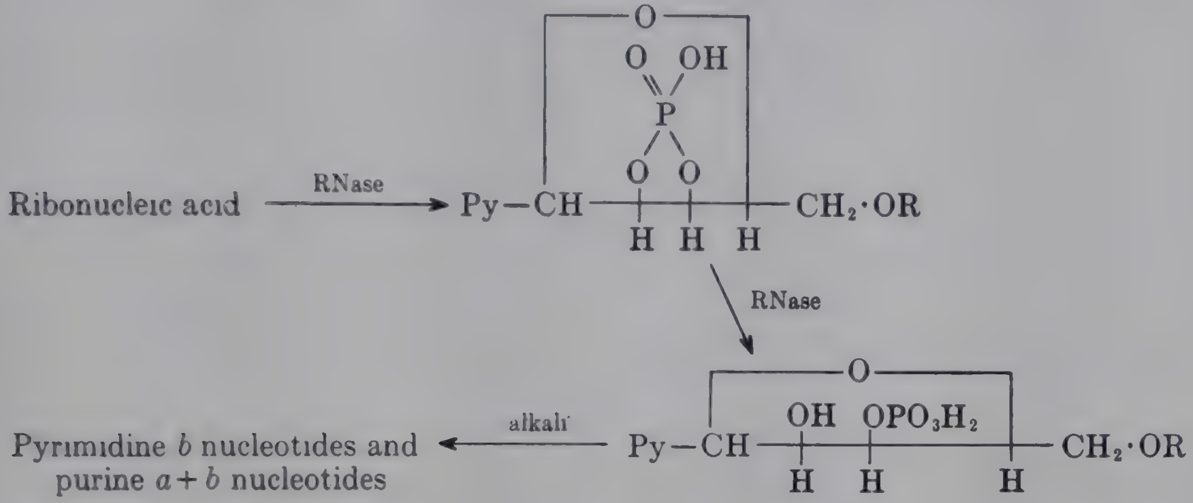
[112] *Inter al.*, G. Schramm, W. Albrecht, and K. Munk, *Z. Naturforsch.* **7b,** 10 (1952)
[113] R. Markham, and J. D. Smith, *Biochem. J.* **52,** 558 (1952).



The evidence so far presented in this section regarding ribonuclease action may now be summarized. The initial reaction of the enzyme evidently occurs specifically at pyrimidine nucleotide sites in the ribonucleic acid molecule. This specificity is discussed in more detail later, but it is supported by the observed failure of ribonuclease to attack the cyclic 2′,3′-phosphates of adenosine and guanosine,[83,109] although it readily attacks their uridine and cytidine analogues. Subject to this specificity, and to the production of only the *b* isomers of pyrimidine mononucleotides, ribonuclease digestion follows a course akin to that of alkaline hydrolysis. The course of ribonuclease hydrolysis and the various degradations described in this section can be conveniently represented in the appended scheme using the earlier postulated 3′,5′-linked polynucleotide to represent ribonucleic acid (in this scheme Py = uracil or cytosine residue, Pu = adenine or guanine residue, and $C_{2'}$—$C_{3'}$—$C_{5'}$ is again used as an abbreviated form of the sugar residue). In this representation of ribonuclease action, the pyrimidine mononucleotide fraction arises from sites in the nucleic acid where two or more pyrimidine nucleotide residues are adjacent, a dinucleotide from sites where a purine nucleotide is flanked on either side by a pyrimidine nucleotide, and larger fragments from positions where several purine nucleotides occur consecutively.[113,114] The production of another type of fragment by ribonuclease action, viz., the so-called "core,"[13,27] is not substantiated by recent work.[96,114] It should be pointed out that although the reactions are

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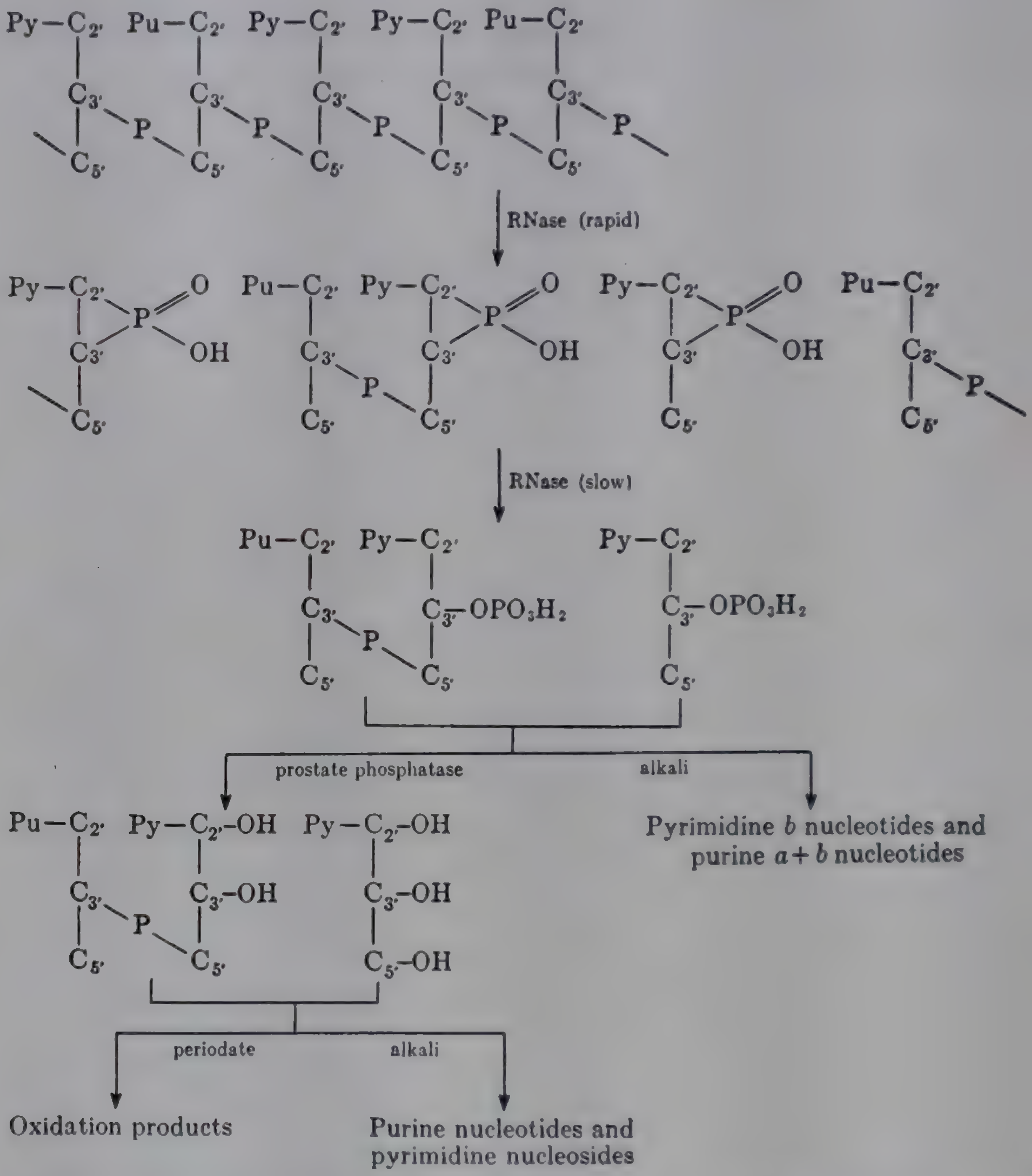
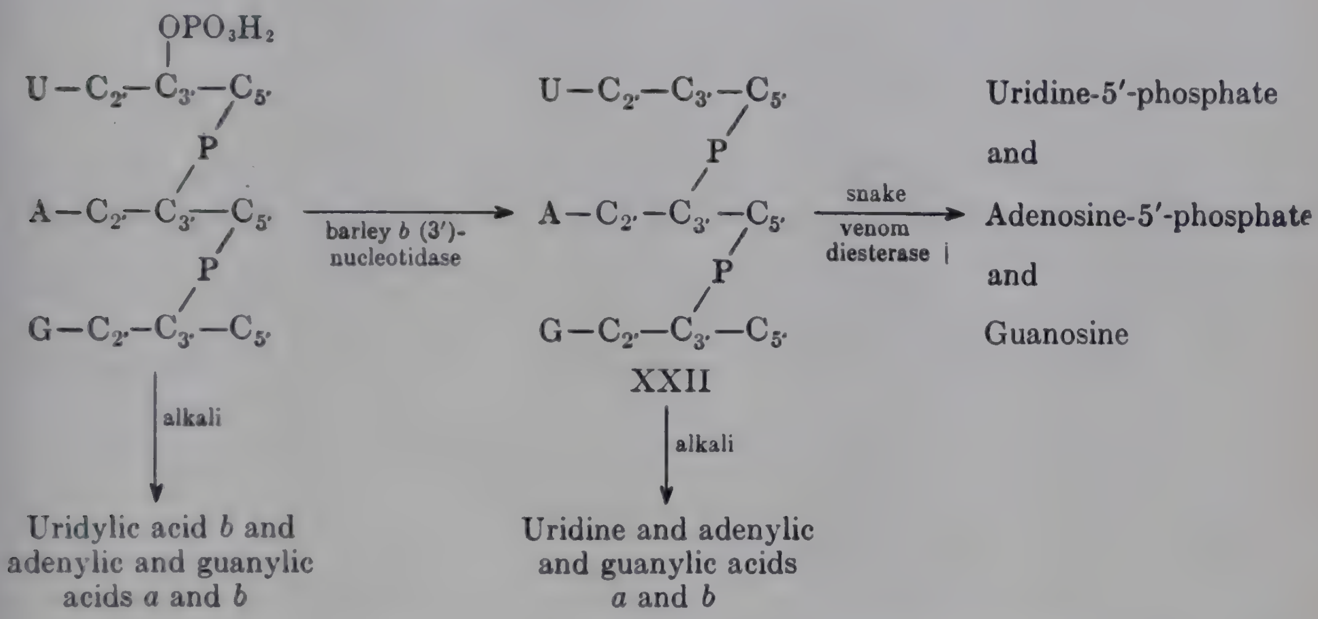
[114] R. Markham and J. D. Smith, *Biochem. J.* **52,** 565 (1952).

represented in the scheme as applying to a straight-chain polynucleotid they are equally readily accommodated on branched-chain structure which fulfil the conditions laid down on the basis of the results of chemica hydrolysis (p. 422).


RNase (rapid)
RNase (slow)
prostate phosphatase
alkali
Pyrimidine b nucleotides and purine a + b nucleotides
periodate
alkali
Oxidation products
Purine nucleotides and pyrimidine nucleosides

6. STRUCTURE OF OLIGONUCLEOTIDES DERIVED FROM RIBONUCLEIC ACIDS

That the general stucture of the oligonucleotides present in ribonuclease digests is correct seems clear from the degradations described above. Their chemistry appears to be wholly consistent with the requirements set out by Brown and Todd[62] from their discussion of chemical hydrolytic mechanisms. It is unfortunate that up to the present, no oligonucleotides have been isolated in substance, apart from the compounds obtained by Merrifield and Woolley[87] from acid hydrolysates of yeast ribonucleic acid. Nevertheless, fractionations of oligonucleotide mixtures in ribonuclease digests have been achieved by paper chromatography and electrophoresis,[113] and by ion-exchange chromatography,[96] which have given solutions of apparently homogeneous substances. Using ion-exchange chromatography, as many as 30–40 distinct elution peaks have been observed, and many of these have been shown to represent solutions of virtually pure oligonucleotides. Using such solutions, the structure of a number of oligonucleotides has been determined. The following example,[96] set out schematically below, will suffice as an example of such a determination carried out on a trinucleotide containing the bases uracil, adenine, and guanine in the ratio 1:1:1 obtained from a ribonuclease digest (in the formulas U, A, and G represent the uracil, adenine, and guanine residues).



Alkaline hydrolysis yielded uridylic acid *b*, together with the mixed *a* and *b* isomers of adenylic and guanylic acid. By removal of the terminal phosphoryl group with phosphomonesterase or barley *b*(3′)-nucleotidase,[115] a product XXII was produced whose molecular weight could be deduced from the ratio of inorganic to total phosphorus. With snake venom diesterase XXII underwent fission at the $C_{3'}$—O—P linkages, giving guanosine and the 5′-phosphates of uridine and adenosine, while with alkali it gave uridine and the mixed purine *a* and *b* nucleotides. Treatment of other oligonucleotides first with phosphomonoesterase, and then with snake venom diesterase, always yielded only nucleoside-5′-phosphates and a nucleoside representing the terminal residue, indicating that all were linear structures since chain-branching would have been expected to lead to other types of breakdown products by the action of snake venom diesterase.

The earlier work of Merrifield and Woolley,[87] in which they isolated and characterized several dinucleotides from acid hydrolysates of yeast ribonucleic acid, is of considerable interest. For structural determination they used methods similar to those just described except that intestinal phosphatase, inhibited with arsenate, was used instead of snake venom diesterase to demonstrate the $C_{5'}$-linkages. Since the formation of their dinucleotides depended on a random fission of the polynucleotide chain, they obtained products containing purine and pyrimidine residues isomeric with those found in ribonuclease digests, but in which the terminal phosphoryl group was attached to the purine nucleoside residue and hence could be further degraded by ribonuclease. Moreover, they were able to separate dinucleotides which were isomeric with one another by virtue of the position (*a* or *b*) of the terminal phosphoryl group, in accordance with prediction from the postulated mechanism of chemical hydrolysis,[62] in which phosphoryl migration must always accompany fission of the diester linkages between individual nucleoside residues in the ribonucleic acid molecule.

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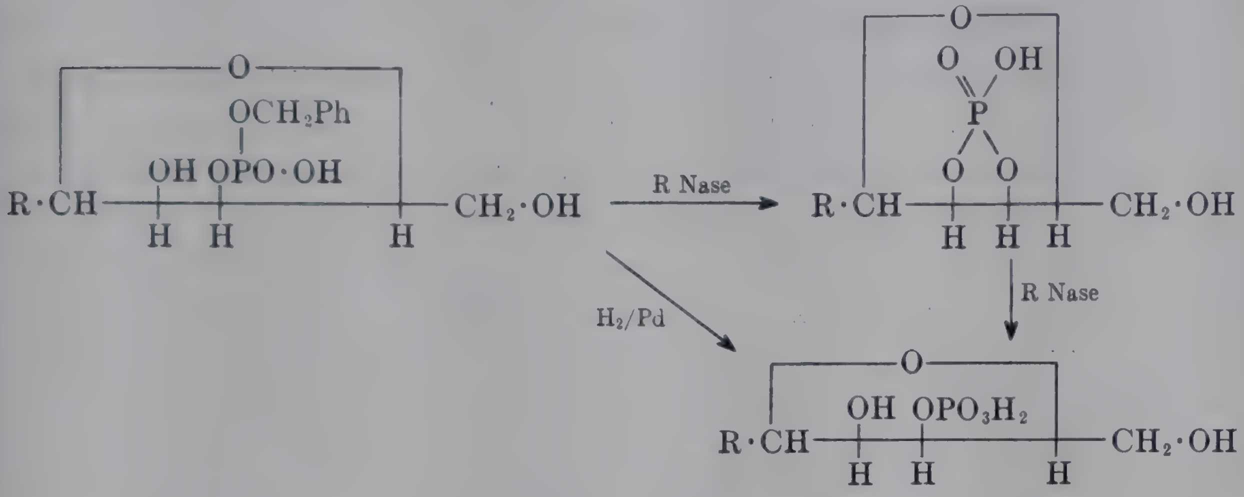
[115] L. Shuster and N. O. Kaplan, *Federation Proc.* **11,** 286 (1952).

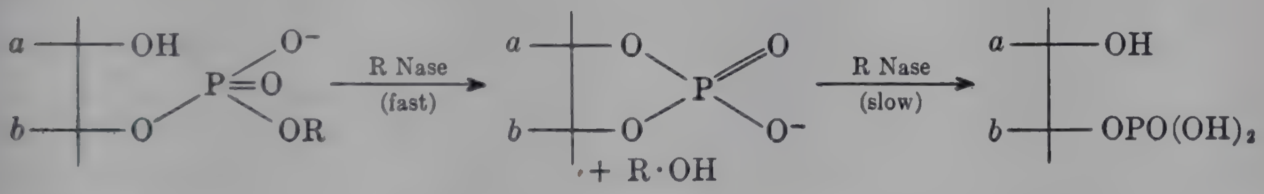
7. Evidence for 3′(*b*)-Linkages in Ribonucleic Acids

In discussing the evidence so far presented on ribonuclease action, the 3′,5′-linked polynucleotide structure has been assumed. However, since degradation with ribonuclease proceeds via intermediates with cyclic 2′,3′-phosphate groupings it is clear that, as in the case of chemical hydrolytic studies, no differentiation can be made between $C_{2'}$ and $C_{3'}$ as a linkage point in the original ribonucleic acids. These could be either 2′,5′- or 3′,5′-linked structures, or might contain both types of linkage. Clearly, finality regarding the linkage requires a decision between $C_{2'}$ and $C_{3'}$.

A solution to this problem was found in the study of the action of enzymes on simple esters of the mononucleotides. Brown and Todd[63] examined the action of ribonuclease on the benzyl esters of the *a* and *b* isomers of the pyrimidine nucleotides. Cytidine benzyl phosphate *b* and uridine benzyl phosphate *b* were converted by ribonuclease into cytidylic acid *b* (cytidine-3′-phosphate) and uridylic acid *b* (uridine-3′-phosphate), hydrolysis proceeding in each case by way of the intermediate cyclic 2′,3′-phosphates. The benzyl esters employed in these experiments were oriented by catalytic hydrogenation to the parent nucleotides, a process unlikely to involve phosphoryl migration. The methyl and ethyl esters were similarly hydrolyzed by ribonuclease. The isomeric esters of the pyrimidine *a* nucleotides were completely unaffected, nor had ribonuclease any action on esters of either *a* or *b* isomers of the purine nucleotides. Not only does this further justify the belief in specificity of ribonuclease for pyrimidine nucleotide ester linkages in ribonucleic acids, but it leads to important conclusions about the linkage position. Since, as shown above, all the internucleotidic linkages involving pyrimidine nucleotide residues are apparently hydrolyzed by ribonuclease, it is clear that the $b(3')$-position of the pyrimidine nucleoside residues is involved exclusively in the internucleotidic linkage. Only in the case of branched-chain structures could the $a(2')$-position be involved and then only as a branching point; this exceptional case will be discussed later.



In discussing the intimate action of ribonuclease, Brown and Todd[63] suggest that the fundamental catalytic action is the formation and subsequent rupture of $C_{a(2')}$—O—P bond as indicated schematically below. The elimination of the residue R as the alcohol ROH is a necessary consequence of this action; the close similarity between ribonuclease and chemical hydrolytic agents is apparent.



This interpretation gives a simple explanation for the apparently twofold action of ribonuclease on ribonucleic acids. The initial "depolymerization" without liberation of acid functions, followed by the slow liberation of acidic secondary phosphoryl groups observed by Chantrenne, Linderstrøm-Lang, and Vandendriessche[116,19] using a dilatometric method is undoubtedly related to the above formation and cleavage of cyclic phosphoryl groups. Vandendriessche[117] has indeed shown that the volume changes observed during the action of ribonuclease on ribonucleic acids are closely parallel to those observed in the ribonuclease hydrolysis of the mononucleotide esters described above.

Although the study of ribonuclease action on mononucleotide esters es-

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[116] H. Chantrenne, K. Linderstrøm-Lang, and L. Vandendriessche, *Nature* **159,** 877, (1947).

[117] L. Vandendriessche, *Acta Chem. Scand.* **7,** 699 (1953).

tablishes $b(3')$ as the linkage point for pyrimidine nucleoside residues, studies with this enzyme can give no information about the corresponding purine nucleoside residues. Evidence on this point comes from studies using other nuclease preparations with different specificities, several of which appear to exist.[100] Volkin and Cohn[110] showed that spleen nuclease prepared according to Maver and Greco[118] yields the *b* isomers of both pyrimidine and purine mononucleotides when it acts upon ribonucleic acid. Further purification of this enzyme preparation by Heppel and Hilmoe[119] gave a product which degrades ribonucleic acids and oligonucleotides, giving high yields of mononucleotides which, in the case of adenylic and guanylic acids, were shown to be the $b(3')$-isomers;[120] since no evidence for intermediate cyclic phosphates was observed, it was concluded that the purine nucleotide residues were linked at the $b(3')$-position in the intact nucleic acid. More definite evidence has been provided by Brown, Heppel, and Hilmoe,[121] who have shown that the same enzyme preparation, as well as others from intestine, potato, and rye-grass, hydrolyze cytidine benzyl phosphate *b* and adenosine benzyl phosphate *b* to the corresponding *b* nucleotides while they have no action on esters of the *a* isomers.

Regardless of the mechanism of spleen nuclease action it is clear that these results, together with the above studies of ribonuclease hydrolysis of nucleotide esters, establish with a high degree of certainty that the internucleotidic linkage in ribonucleic acids involves the $b(3')$-position and not the $a(2')$-position in both purine and pyrimidine nucleoside residues. Assuming the validity of the constitutions proposed for the mononucleotides, i.e., that the *b* isomers are the 3′-phosphates, the ribonucleic acids must, on the evidence presented, be considered to be polynucleotides in which the individual nucleoside residues are joined one to the other by phosphodiester linkages between the 3′- and 5′-positions as indicated in structure XVI.

Although the oligonucleotides produced by ribonuclease action appear to be unbranched, this fact cannot of itself be accepted as evidence that intact ribonucleic acids are linear polynucleotides. To complete our discussion it is necessary to consider the question of chain-branching.

8. Chain-Branching in Ribonucleic Acids

There has been much discussion in the past about branched-chain as distinct from linear structures for the ribonucleic acids. This involved, in the main, discussion of branching from the main chain by incorporation of phosphotriester linkages.[10] However, with the advent of an adequate explanation of the hydrolytic behavior of ribonucleic acids it became possible to define with some certainty the possible types of branching which might be considered. As has already been briefly mentioned, Brown and Todd,[62] starting from the requirement that ribonucleic acids are hydrolyzed to mononucleotides under mild alkaline conditions, pointed out that in addition to branching on phosphorus, branching by attachment of polynucleotide chains to $C_{2'}$ in one of the nucleoside residues in a main polynucleotide chain of type XVI could be considered, provided that the attachment was through a normal phosphodiester group attached to $C_{3'}$ (or $C_{2'}$) in the first nucleoside residue of the branching chain; only in this way could alkali-lability be maintained. $C_{5'}$ could not be considered as the point of linkage in the first residue of the branch as the result would be an alkali-stable system, cyclization either in that residue or in the residue forming the branch-point in the main chain being impossible. These authors therefore proposed a general structure of the type XXIII for branched ribonucleic acids. In this structure the branches can be extended following the normal $C_{3'}$—$C_{5'}$ sequence as in the main chain. The incorporation of a certain number of branches involving phosphotriester linkages into such a structure would present no problem provided, again, that the $C_{3'}$—$C_{5'}$ sequence was followed in the branches.

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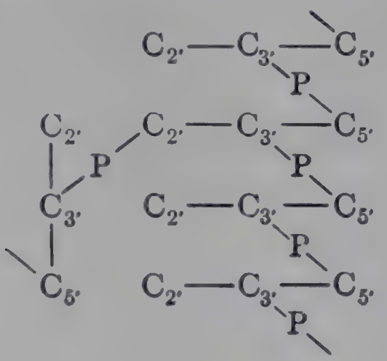
[118] M. E. Maver and A. E. Greco, *J. Biol. Chem.* **181,** 861 (1949).
[119] L. A. Heppel and R. J. Hilmoe, *Federation Proc.* **12,** 217 (1953).
[120] L. A. Heppel, R. Markham, and R. J. Hilmoe, *Nature* **171,** 1152 (1953).
[121] D. M. Brown, L. A. Heppel, and R. J. Hilmoe, *J. Chem. Soc.,* **1954,** 40.



XXIII

Some support for the branched-chain structure XXIII was provided by methylation studies.[122,123] Methylation of yeast ribonucleic acid, followed by hydrolysis and separation of the mixture of sugar derivatives obtained, yielded ribose as well as its mono- and dimethyl derivatives. The presence of ribose was taken to indicate triply-substituted ribofuranose residues in the nucleic acid.[123] It is, however, doubtful if the method of methylation can be safely applied in this field on account of the lability of the internu-

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[122] A. S. Anderson, G. R. Barker, and K. R. Farrar, *Nature* **163**, 445 (1949).

[123] A. S. Anderson, G. R. Barker, J. M. Gulland, and M. V. Lock, *J. Chem. Soc.* **1952**, 369.

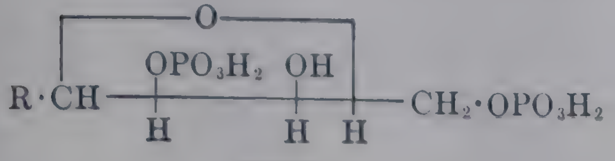
cleotidic linkages in the presence of alkaline reagents and the difficulty of assessing the completeness of methylation.[123a]

In discussing structure XXIII, Brown and Todd[62] suggested that the rapid production of pyrimidine mononucleotides during ribonuclease digestion might be explained by postulating that branches were short and frequent—perhaps consisting of only one nucleoside unit in some cases—and consisted of pyrimidine nucleotide residues. Fission at the $C_{2'}$—O—P bond at the branch-point would yield pyrimidine nucleotides (via cyclic phosphates) readily. Rather similar suggestions based, however, on branched structures of undefined type, were also advanced by Carter and Cohn[99] and Magasanik and Chargaff.[27] The more recent developments in our knowledge of ribonuclease action make it clear that it is not necessary to postulate branches as the source of the pyrimidine mononucleotides, but it has made it evident that in any branching of the type shown in XXIII the first residue in the branch must be a pyrimidine nucleotide;[62] if it were not, branched oligonucleotides would be formed.

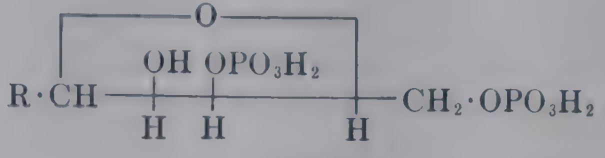
Cohn and Volkin[124] have brought forward further evidence bearing on the problem of branching from studies using snake venom diesterase, and have discussed their results in terms of structure XXIII. Following the work of Gulland and Jackson[39] with snake venoms which contain diesterases and 5′-nucleotidases, they were able to confirm that, acting on ribonucleic acids, large amounts of inorganic phosphate are liberated. In addition, however, they showed that cytidine diphosphate and uridine diphosphate were produced simultaneously in an amount corresponding to about 30% of the pyrimidine content of the nucleic acid. That the inorganic phosphate originated mainly in nucleoside-5′-phosphates was shown by experiments using venom which had been freed of 5′-nucleotidase by the method of Hurst, Little, and Butler.[93] This purified preparation acting on ribonucleic acids from calf-liver, thymus, and yeast, liberated very little inorganic phosphate but yielded large amounts of all four nucleoside-5′-phosphates, the pyrimidine nucleoside diphosphates, some nucleosides (mainly purine), and about 10% of pyrimidine *b* nucleotides. The diphosphates were shown by enzymic degradation to be mixtures of cytidine-2′,5′- and cytidine 3′,5′-diphosphate (XXIVa and b; R = cytosine residue) and the corresponding uridine diphosphates (XXIVa and b; R = uracil residue), structurally analogous to the adenosine diphosphates obtained by degradation of triphosphopyridine nucleotide[125] and coenzyme A.[126]

[123a] More recent work (D. M. Brown, D. I. Magrath, and A. R. Todd, *J. Chem. Soc.* **1954,** 1442) has shown that during the methylation of uridylic acid *b*, phosphoryl migration occurs; the method is therefore unlikely to afford reliable evidence when applied to polynucleotides since internucleotidic bond fission must on this evidence be expected to accompany methylation.

[124] E. Volkin and W. E. Cohn, *J. Biol. Chem.*, **203,** 319 (1953).



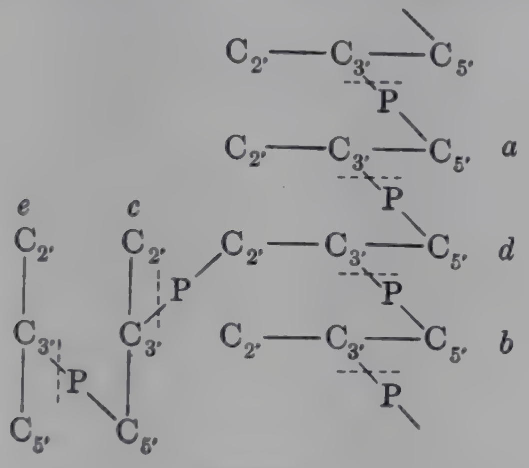
XXIVa



XXIVb

The pyrimidine nucleoside diphosphates do not appear to be produced from the end-groups of the ribonucleic acids bearing monoesterified phosphate since they are obtained in undiminished yield from nucleic acids previously treated with bone phosphomonoesterase. This enzyme removed about 10% of bound phosphate, considered on other grounds to represent end-group monoesterified phosphate. Oligonucleotides produced by ribonuclease action from the nucleic acids were attacked very slowly, if at all, by the purified venom.

Volkin and Cohn[124] explain these observations on the basis of a branched-chain structure based on XXIII which can be represented by XXIIIa. They



XXIIIa

suggest that the diesterase causes fission of the internucleotidic linkages at the $C_{3'}$—O—P bonds (i.e., at the broken lines in XXIIIa) liberating nucleoside-5′-phosphates (from residues *a*, *b*, and *c*) as in the case of the polydeoxyribonucleotides.[93] The diphosphates would arise from branch points (residue *d*) and the (purine) nucleosides from the terminal residue of the branch (residue *e*). The purine nucleoside produced in their experiments was in fact equivalent in amount to the nucleoside diphosphates. For this explanation to hold good, they had to postulate that the residue at the branching point (*d*) is a pyrimidine nucleoside residue. The first residue (*c*) in the branch must also be a pyrimidine nucleoside residue to accomodate the results of ribonuclease studies, as already pointed out. The small amount of pyrimidine *b* mononucleotide present in the venom digests was tentatively regarded[124,127] as originating in side chains consisting of only one nucleotide residue attached through triply-esterified phosphorus, as in the early structural proposals of Gulland and his colleagues[10]. Their own and other[128] observations indicate that some 10% of the phosphoryl groups in ribonucleic acids are singly esterified.

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[125] A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.* **186,** 557 (1950).

[126] T. P. Wang, L. Shuster, and N. O. Kaplan, *J. Am. Chem. Soc.* **74,** 3204 (1952).

While these experiments favor a branched-chain structure for ribonucleic acids, it would be unwise to rely entirely on evidence from only one type of degradation and further confirmation would be welcome. In any case, it is not impossible that among the natural ribonucleic acids both linear and branched-chain polynucleotides may occur; such a possibility has, in fact, been suggested[129] and certainly there appears to be no evidence against it at the present time.

9. NUCLEOTIDE SEQUENCE IN POLYRIBONUCLEOTIDES

In previous pages the problem of the nature and position of the internucleotidic linkage has been discussed in the light of recent work. A clear picture has emerged in which the nucleoside residues in the ribonucleic acids are joined at their 3′- and 5′-positions through phosphodiester linkages; the possible types of branching have been indicated and evidence for their occurrence has been reviewed. In brief, it appears that for a straight-chain ribonucleic acid the general structure XVI will apply, and for branched-chain acids structure XXIII, with the possible addition of some branching through phosphotriester linkages. The degree to which branching may occur in ribonucleic acids generally is not yet established, but this does not affect the general structures as far as the internucleotidic linkage is concerned.

In discussing these structures little attention has been paid to the order in which the four different nucleotide residues occur in the polynucleotide chain. It has, however, been pointed out that in a branched structure XXIII the first residue in the branch must be a pyrimidine nucleotide residue, and that in the more specific structure XXIIIa the branching point in the main chain must also be a pyrimidine nucleotide residue. Only in the case of the small oligonucleotides has residue sequence been determined and the results of such determinations do not shed a great deal of light on the situation in the intact nucleic acids. Nucleotide sequence in ribonucleic acids cannot be simply elucidated by partial degradation to

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[127] W. E. Cohn, D. G. Doherty, and E. Volkin, *Phosphorus Metabolism* **2,** 339 (1952).

[128] G. Schmidt, M. Seraidarian, K. Seraidarian, and S. J. Thannhauser, *Federation Proc.* **11,** 283 (1952).

[129] G. Schramm and B. von Kerékjártó, *Z. Naturforsch.* **7b,** 589 (1952).

polynucleotides, followed by structural determinations on these and reconstruction to yield a unique solution. The fact that only four mononucleotides are involved makes this type of approach in some respects less likely to succeed than in the case of the proteins, as is evident when one considers the results obtained by analyzing the oligonucleotides isolated from ribonuclease digests of ribonucleic acids.[96,113,129a] All the possible residue arrangments are found in the di- and trinucleotides (with terminal pyrimidine residues) obtained in this way, and there is little reason to believe that the same will not also hold for the mixture of larger nondialyzable fragments which are obtained. The problem would be even more complicated if, as Markham and Smith[114] suggest, ribonucleic acids are to be regarded as mixtures of large numbers of relatively small molecules rather than single species of very high molecular weight. These authors arrived at this suggestion on the basis of a method of end-group determination depending on removal of terminal monoesterified phosphate with prostatic phosphatase; using it, they found all four nucleotides as end-groups in addition to nucleotides bearing a cyclic phosphoryl group. Their conclusions seem open to criticism, however, firstly because chain-branching, which they do not consider, could account for increased numbers of end-groups, and secondly because the presence of traces of diesterases in their enzyme preparations would lead to high apparent figures for end-groups and correspondingly low estimates of molecular weight.

At the present time, available evidence does not indicate anything more than a rather random sequence of residues, and it seems clear that progress must depend (a) on the development of methods of stepwise degradation and (b) some certainty as to the individuality of a given ribonucleic acid—clearly if it were a mixture of different molecular species[130] efforts to determine sequence would be useless. A method of stepwise degradation is in any case of considerable value, since quite apart from its use on intact ribonucleic acids, such a method is necessary if the structure of the larger oligonucleotides is to be determined. The essential requirement for such a method is that the terminal internucleotidic linkage in a chain should be rendered more labile than any other similar linkage in the molecule so as to permit removal (and identification) of that residue, leaving the remainder of the polynucleotide intact and ready for a repetition of the same process. The lability of the internucleotidic linkage severely limits the choice of possible methods. Brown, Fried, and Todd[131] have proposed a method

[129a] The recent demonstration (L. A. Heppel and P. R. Whitfeld, *Proc. Biochem. Soc.* **56**, ii, 1954; L. A. Heppel, P. R. Whitfeld, and R. Markham, *ibid.* **56**, iii, 1954) that ribonuclease acts reversibly must render ineffectual any attempt to derive the structure of a polynucleotide from a consideration of the products of its action.

[130] V. Desreux and J. M. Ghuysen, *Bull. soc. chim. Belges* **60**, 410 (1951).

[131] D. M. Brown, M. Fried, and A. R. Todd, *Chemistry & Industry* **1953**, 352.

which meets the above requirements and which depends on the known lability towards alkali of esters, and in particular, phosphates of β-keto and β-aldehydo alcohols. Such phosphates readily undergo an elimination reaction under these conditions;[132] the reaction is illustrated by the conversion of glyceraldehyde-3-phosphate to lactic acid under very mild alkaline and pyruvaldehyde under acid conditions.[133,134] In model experiments,[131] adenosine-5′-phosphate and adenosine-5′ benzyl phosphate were oxidized with periodic acid to give dialdehydes of formula XXV (Ad = adenine residue, R = H or $CH_2C_6H_5$) in which the phosphate residue is attached in the β-position to one of the aldehyde groups. In accordance with expectation, these products were extremely labile to alkali, the phosphoryl (or benzylphosphoryl) group being rapidly removed at room temperature even at pH 10.5; under these conditions the unoxidized nucleotides, as well as ribonucleic acids,[134a] are completely stable. Since adenosine-5′ benzyl phosphate is structurally analogous to a polynucleotide (in the latter the benzyl group is replaced by a polynucleotide chain) the potentialities of this observation are evident. If formula XXVI represents a polynucleotide made up of n nucleotides, removal of the terminal phosphoryl groups by means of a phosphomonoesterase would yield XXVII, which, containing a free α-glycol system, would be oxidized by periodic acid to XXVIII. By analogy with oxidized adenosine-5′-phosphate, mild alkaline treatment at pH 10.5 would be expected to remove the oxidized terminal nucleoside residue (which could be identified by various methods) yielding XXIX, which is a polynucleotide containing $n - 1$ residues but otherwise exactly like the original XXVI; on this product the whole process could be repeated.

Although the method has not yet been applied to a large polynucleotide, there is no reason to doubt its validity in such a case, especially as it has been found applicable to dinucleotides where $n = 2$.[135] At the present time this method represents the only reasoned approach to determination of nucleotide sequence; if successful in its application it should also provide definite information on the nature and extent of any branching which exists in a given polynucleotide.

It is of interest to note that the polynucleotide XXVI can be regarded as a polymer in which the monomeric units are nucleoside-3′-phosphates. It would be equally reasonable (and it would not affect the previous discussion) if it were represented as XXVIa, in which the monomeric units are nucleoside-5′-phosphates. To which of these two types the natural nucleic acids belong is at present unknown. That apparently successful end-group

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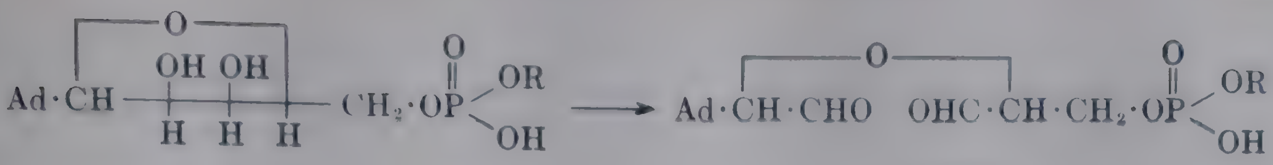
[132] R. P. Linstead, L. N. Owen, and R. F. Webb, *J. Chem. Soc.* **1953**, 1211.

[133] O. Meyerhof and K. Lohmann, *Biochem. Z.* **271,** 89 (1934).

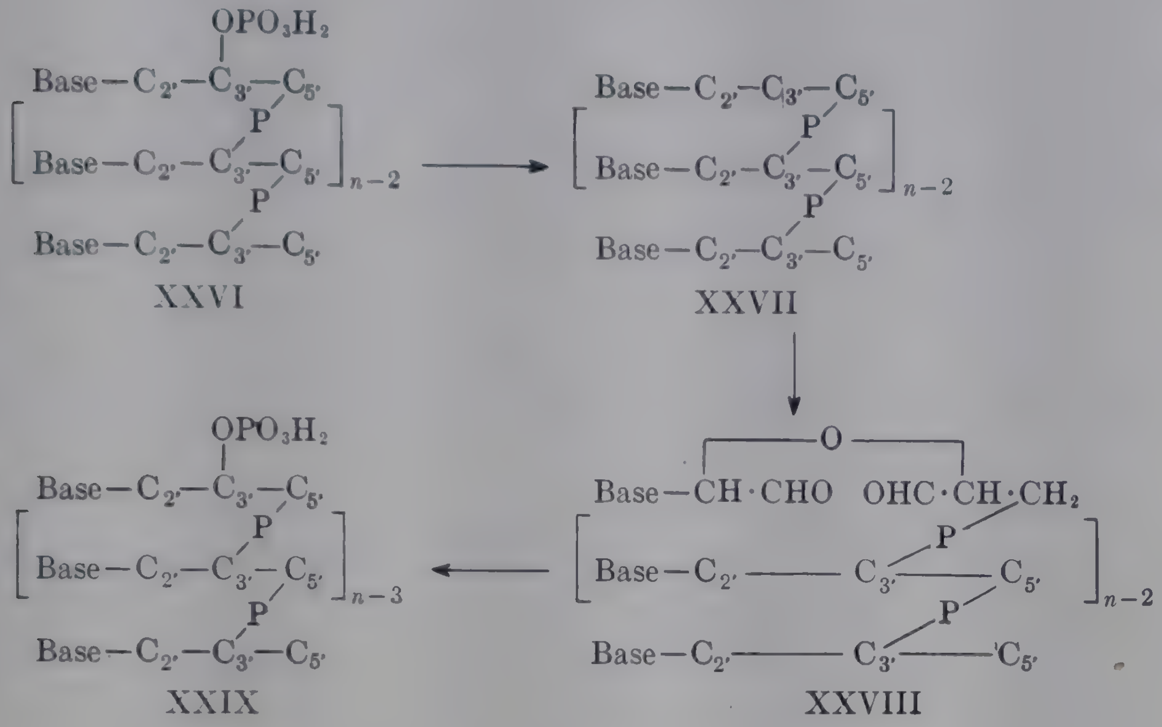
[134] E. Baer and H. O. L. Fischer, *J. Biol. Chem.* **150,** 223 (1943).

[134a] Cf. also C. A. Zittle, *J. Franklin Inst.* **242,** 221 (1946).

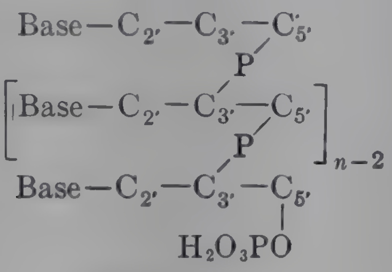
[135] P. R. Whitfeld and R. Markham, *Nature* **171,** 1151 (1953).



XXV



XXVI XXVII XXVIII XXIX



XXVIa

determinations have been made using phosphomonoesterase to remove a terminal $C_{3'}$-phosphoryl group is not valid evidence of XXVI unless it can be shown that the nucleic acid is completely intact; any partial degradation of either type of polynucleotide would inevitably lead to structures of type XXVI, on the basis of the accepted mechanism of hydrolytic breakdown.[135a] A similar uncertainty exists in the case of the deoxyribonucleic acids.

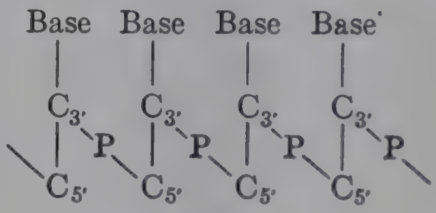
III. Structure of the Deoxyribonucleic Acids

The deoxyribonucleic acids have generally been considered on titrimetric data to be essentially high-molecular-weight polynucleotides which are

[135a] Since this review was written, Markham, Matthews, and Smith (*Nature* **173**, 537, 1954) have shown that the nucleic acids of tobacco mosaic virus and potato virus X belong in large proportion to the class represented by XXVIa; alkaline hydrolysis yields nucleoside-2′(and -3′),5′-diphosphates and nucleosides originating from terminal residues. Other nucleic acids, e.g., from turnip yellow mosaic virus, appear to conform to structures of type XXVI, although, as indicated above, this could be a reflection of their degraded state.

substantially unbranched, and in which the individual nucleotides are joined by phosphodiester linkages.[20] Certainly most of the available evidence[136] is in agreement with this although there has been an isolated suggestion,[22] on titrimetric results, that chain-branching through phosphotriester linkages (about once in every 10–20 linkages) occurs and some slight degree of branching has been also suggested from work using light-scattering techniques.[137] These suggestions are at present unsubstantiated by other physical studies, and no evidence for branching has been found in degradative studies. The observations on which they rest may be susceptible of other interpretations in the light of recent structural models proposed by other workers[138,139] in which hydrogen-bonding between associated polynucleotide chains plays a major role. For the purposes of the present discussion, therefore, the deoxyribonucleic acids will be considered as essentially unbranched polynucleotides.

In their discussion of the hydrolytic behavior of the nucleic acids, Brown and Todd[62] adopted for deoxyribonucleic acids a general structure of type XXX (Base—$C_{3'}$—$C_{5'}$ represents a nucleoside unit), in which the deoxynucleoside units are linked by 3′,5′-phosphodiester groupings. This structure, they pointed out, was in accord with the alkali-stability of deoxyribonucleic acids, and it is also borne out by the results of chemical and enzymic degradation.



XXX

It was claimed many years ago by Levene and Jacobs[140] that acid hydrolysis of thymus deoxyribonucleic acid under fairly vigorous conditions yielded, among other products, two substances which were diphosphates of the two pyrimidine deoxyribonucleosides, thymidine and deoxycytidine; this claim was subsequently disputed by other workers.[141] Dekker, Michelson, and Todd[142] have reinvestigated this matter using deoxyribonucleic acid from herring sperm and have vindicated the claim of Levene and Jacobs.[140,143,144]

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[136] *Inter al.*, R. Signer and H. Schwander, *Trans. Faraday Soc.* **46,** 790 (1950).

[137] M. E. Reichmann, R. Varin, and P. Doty, *J. Am. Chem. Soc.* **74,** 3203 (1952); P. Doty and B. H. Bunce, *ibid.* **74,** 5029.

[138] L. Pauling and R. B. Corey, *Nature* **171,** 346 (1953).

[139] J. D. Watson and F. H. C. Crick, *Nature* **171,** 737 (1953).

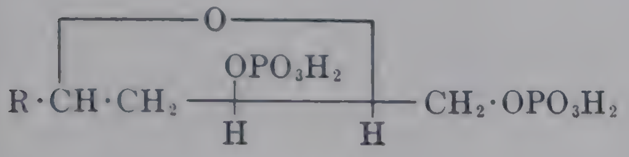
[140] P. A. Levene and W. A. Jacobs, *J. Biol. Chem.* **12,** 411 (1912).

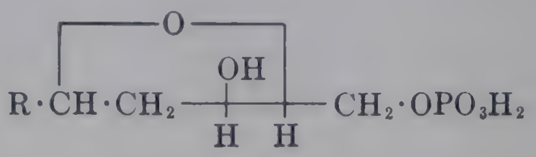
[141] H. Bredereck and G. Caro, *Z. physiol. Chem.* **253,** 170 (1938).

[142] C. A Dekker, A. M. Michelson, and A. R. Todd, *J. Chem. Soc.* **1953,** 947.

[143] See also P. A. Levene, *J. Biol. Chem.* **48,** 119 (1921); **126,** 63 (1938).

They were able to isolate from acid hydrolysates thymidine-3′,5′-diphosphate (XXXI; R = thymine residue) and deoxycytidine-3′,5′-diphosphate (XXXI; R = cytosine residue) and to establish their structure by synthesis; they also obtained some evidence for the presence of small amounts of the corresponding 3′,5′-diphosphate of 5-methyldeoxycytidine in their hydrolysates. The isolation of these substances is a clear indication that both the 3′- and 5′-positions in the deoxyribonucleosides are involved in the internucleotidic linkage and emphasizes the difference between the hydrolytic mechanism which operates in ribonucleic and deoxyribonucleic acids.


XXXI


XXXII

Chemical hydrolysis of deoxyribonucleic acids is not a satisfactory method for obtaining simple mononucleotides mainly on account of the lability of the glycosidic linkage in the purine deoxyribonucleotides to acid, and the resistance of the normal phosphodiester group to alkaline hydrolysis. Klein and Thannhauser,[145] however, using an arsenate-inhibited intestinal phosphatase, succeeded by enzymic hydrolysis in isolating four nucleotides (deoxyadenylic, deoxyguanylic, deoxycytidylic, and thymidylic acid); Volkin, Khym, and Cohn[146] also described their separation from similar hydrolysates. Similarity to the ribonucleoside-5′-phosphates in their ion-exchange characteristics and their dephosphorylation[147] by a specific 5′-nucleotidase[148] indicated that, contrary to earlier assumptions, they were the 5′-phosphates of the respective nucleosides (XXXII; R = purine or pyrimidine residue). Conclusive proof for this formulation has been provided at least in the case of the pyrimidine nucleotides by identification of the natural substances through direct comparison with thymidine-5′-phosphate (XXXII; R = thymine residue) and deoxycytidine-5′-phosphate (XXXII; R = cytosine residue) prepared by unambiguous methods by Michelson and Todd.[149] The almost quantitative recovery (*ca.* 92%) of the deoxyribonucleoside-5′-phosphates from snake venom diesterase hydrolysates of deoxyribonuclease-treated deoxyribonucleic acids[30,93] shows that

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[144] See also S. J. Thannhauser and B. Ottenstein, *Z. physiol. Chem.* **114,** 39 (1921); S. J. Thannhauser and G. Blanco, *ibid.* **161,** 116 (1926).

[145] W. Klein and S. J. Thannhauser, *Z. physiol. Chem.* **218,** 173 (1933); **224,** 252 (1934); **231,** 96 (1935).

[146] E. Volkin, J. X. Khym, and W. E. Cohn, *J. Am. Chem. Soc.* **73,** 1533 (1951).

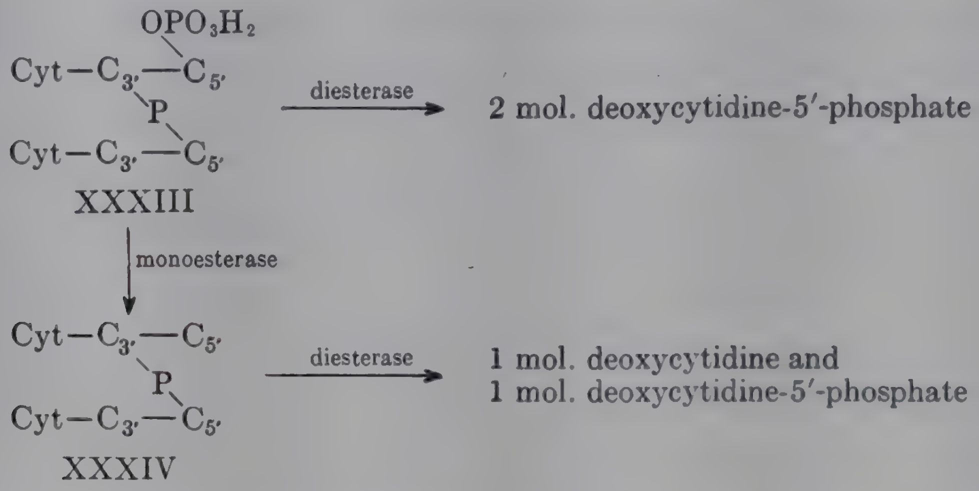
[147] C. E. Carter, *J. Am. Chem. Soc.* **73,** 1537 (1951).

[148] L. A. Heppel and R. J. Hilmoe, *J. Biol. Chem.* **188,** 665 (1951).

[149] A. M. Michelson and A. R. Todd, *J. Chem. Soc.* **1953,** 951; *1954,* 34.

all, or almost all, the internucleotidic linkages must involve the 5′-position of a nucleoside residue. These facts all point clearly to an essential structure of type XXX.

Evidence from other enzymic studies supports this conclusion. The mode of action of the crystalline deoxyribonuclease[29] is not yet understood, but several workers[150-152] have shown that a number of oligonucleotides of varying size are produced when it acts on deoxyribonucleic acids. The use of electrophoretic,[151,152] paper chromatographic,[152] and ion-exchange[153] methods have permitted the separation and identification of some of these oligonucleotides. Sinsheimer and Koerner[153] have described two dinucleotides, one containing two cytosine residues and the other an adenine and a cytosine residue, and have determined their structures by enzymic hydrolysis. The method used is indicated below, using the deoxycytidine dinucleotide XXXIII as an example. Phosphomonoesterase treatment yielded the dinucleoside phosphate XXXIV[154] which in turn yielded, with snake venom diesterase, both deoxycytidine-5′-phosphate and the free nucleoside, deoxycytidine. Direct enzymic hydrolysis of the original dinucleotide (XXXIII), on the other hand, yielded only deoxycytidine-5′-phosphate, indicating that a phosphoryl group is attached at $C_{5'}$ in both residues.



1. Nucleotide Sequence in Deoxyribonucleic Acids

Although, as the above discussion shows, the nature and position of the main internucleotidic linkage in deoxyribonucleic acids seems clear, the problem of nucleotide sequence remains. [Cf. Chargaff, Chapter 10.] On this no definite statement can be made. As yet no method of stepwise degradation comparable to that proposed for ribonucleic acids[131] has been evolved.

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[150] W. G. Overend and M. Webb, *J. Chem. Soc.* **1950,** 2746.

[151] A. H. Gordon and P. Reichard, *Biochem. J.* **48,** 569 (1951).

[152] J. D. Smith and R. Markham, *Nature* **170,** 120 (1952).

[153] R. L. Sinsheimer and J. F. Koerner, *J. Am. Chem. Soc.* **74,** 283 (1952).

[154] Cf. also J. D. Smith and R. Markham, *Biochim. et Biophys. Acta* **8,** 350 (1952).

No attempts have been made to piece together information based on the structure of oligonucleotides found in deoxyribonuclease digests; for reasons similar to those discussed in connection with the ribonucleic acids (p. 437) it is, in any case, unlikely that such attempts would lead to a unique solution. Chargaff and his co-workers, however, have sought to ascertain whether there is any recognizable order in the nucleotide sequence of typical deoxyribonucleic acids. Thus Zamenhof and Chargaff[26,155,156] have studied the distribution of purine and pyrimidine bases in the dialyzable and nondialyzable fractions of deoxyribonuclease digests. Their results suggest a very complex pattern; it should be remembered, of course, that the as yet unknown specificity of deoxyribonuclease and the effect of different dialysis rates of the fragments must be taken into account in drawing any definite conclusions from such studies. The separation of six dinucleotides from such digests[152] seems to indicate that no simple regularity is to be expected in the nucleotide distribution.

When deoxyribonucleic acids are subjected to mild acid hydrolysis, materials are formed which were formerly described by the generic term "thymic acid."[157] Although most of the earlier preparations were highly degraded, Chargaff and his co-workers have reinvestigated their preparation and obtained an interesting group of substances of molecular weight *ca.* 15,000 which they term apurinic acids.[158] As their name suggests, they are produced by removal of all purine residues from the polynucleotide, through fission of the labile purine *N*-glycosidic linkages, and thus have deoxyribose phosphate residues in place of the purine nucleotide residues originally present in the nucleic acid. It is obvious that such products are of great structural interest and their hydrolysis would merit close study.

Relatively vigorous acid hydrolysis of deoxyribonucleic acids yields, as already indicated, the 3′,5′-diphosphates of thymidine and deoxycytidine. Brown and Todd[62] originally suggested that a certain amount of these diphosphates might be expected as a result of random fission of internucleotidic linkages. Dekker, Michelson, and Todd,[142] however, have commented on the unexpectedly large amounts of the diphosphates in acid hydrolysates and suggest that they owe their origin to some other mechanism operating at those positions in the polynucleotide chain where pyrimidine and purine residues are adjacent to one another in view of the above-mentioned evidence[157] that the initial action of acid is to remove purine residues from deoxyribonucleic acids. A probable mechanism is that discussed by Brown, Fried, and Todd[131] as a basis for stepwise degradation of polyribo-

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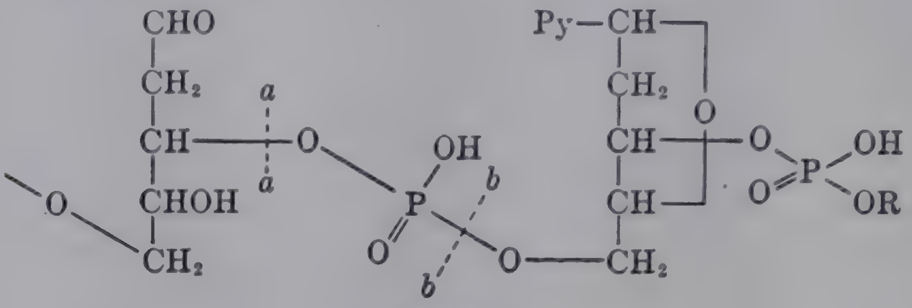
[155] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **178,** 531 (1949).

[156] S. Zamenhof, *Phosphorus Metabolism* **2,** 301 (1952).

[157] See footnote 158 for bibliography.

[158] C. Tamm, M. E. Hodes, and E. Chargaff, *J. Biol. Chem.* **195,** 49 (1952).

nucleotides, i.e., the tendency of phosphates of β-aldehydo alcohols to undergo elimination reactions (cf. p. 437). The initial action of acid must lea to products of the apurinic acid type. If we consider a section (XXXV R = next residue in the chain) of such a molecule, ready elimination shoul occur at $C_{3'}$ of the deoxyribose residue (here written in the aldehyde form as indicated by the broken line *a a*.



XXXV

The alternative fission i.e., at the point indicated by the broken line *b* in XXXV, which would result from intervention of a cyclization between $C_{3'}$ and $C_{4'}$ in the deoxyribose residue, analogous to the mechanism involved in ribonucleic acid hydrolysis,[62] is less likely; if it occurred, one would expect nucleosides or nucleotidic materials deficient in phosphorus to be produced. If the next residue (R) in the formula (XXXV) represents a pyrimidine nucleoside residue, a product larger than a mononucleotide will be first formed, but in the event of R being another deoxyribose residue (i.e., the original site of a purine nucleoside residue) production of a pyrimidine nucleoside diphosphate might be expected. It is clear that further detailed investigation of the products of acid hydrolysis is desirable.

Tamm *et al.*[159] have found that apurinic acids are rapidly degraded in alkaline solution to give a mixture of dialyzable and nondialyzable products. The nondialyzable fraction contains *ca.* 85% of the pyrimidine nucleotide residues but only some 40% of the deoxyribose phosphate residues present in the starting material. They interpret this degradation by invoking the cyclization mechanism mentioned above in discussing the breakdown of XXXV, and from their findings they have deduced that calf thymus deoxyribonucleic acid consists of a chain in which sections of the chain containing principally pyrimidine nucleotides are followed by stretches in which purine nucleotides predominate. There appears to be some discrepancy between this conclusion and the isolation of large amounts of pyrimidine nucleoside diphosphates from acid hydrolysates if these latter substances arise by the mechanism discussed above. The appearance of the diphosphates suggests a fairly even or random distribution of purine and pyrimidine nucleotide residues. A likelier mechanism for the alkaline degradation of apurinic acid is that discussed above for structure XXXV, in which fission at the broken line *a a* by an elimination reaction or by hydrolysis is a major factor; this mechanism would lead to products in which the pyrimidine nucleoside residues retain phosphorus in excess of the mononucleotide ratio (1:1), e.g., nucleoside diphosphates, dinucleoside triphosphates, etc. The low rate of dialysis of the pyrimidine-containing fragments of Tamm *et al.*[159] against distilled water might be explained by the association of low molecular weight with high ionic charge.[114] At present it is not possible to resolve these matters, and further evidence bearing on nucleotide sequence is required.

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[159] C. Tamm, H. S. Shapiro, R. Lipshitz, and E. Chargaff, *J. Biol. Chem.* **203,** 673 (1953).

CHAPTER 13

The Physical Properties of Nucleic Acids

D. O. JORDAN

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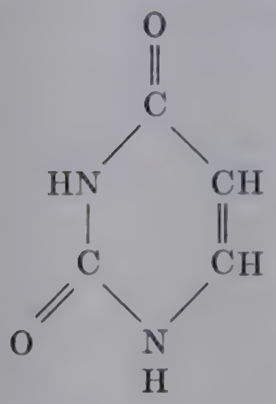
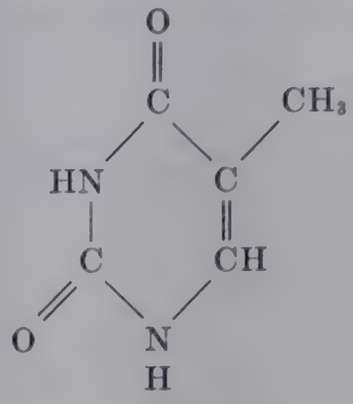
I. Pyrimidines, Purines, Nucleosides, and Nucleotides

1. STRUCTURE DETERMINATIONS BY X-RAY DIFFRACTION

a. Pyrimidines

The full details of the structures of uracil (I), thymine (5-methyluracil, II), cytosine (III), and 5-methylcytosine (IV), the pyrimidines known to occur in nucleic acids [cf. *Bendich*, Chapter 3], have not been established by X-ray diffraction. Some conclusions may be drawn concerning their structures, however, from those of some related pyrimidine derivatives which have been studied. Clews and Cochran[1,2] have determined the struc


I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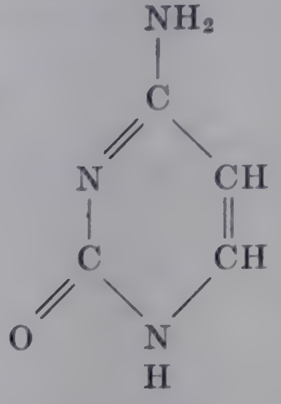
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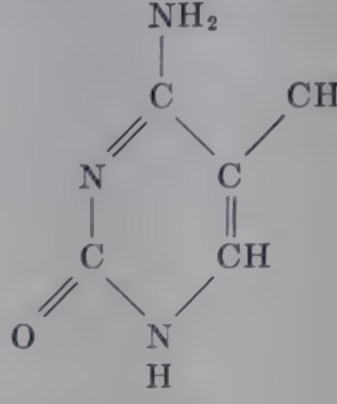
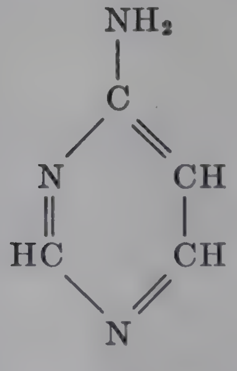
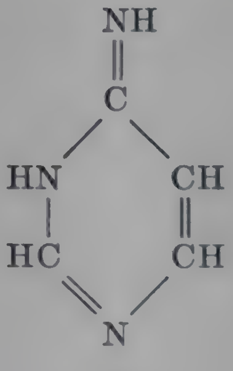

IV

ture of 2-amino-4-methyl-6-chloropyrimidine, 2-amino-4,6-dichloropyrimidine, 4-amino-2,6-dichloropyrimidine, and 5-bromo-4,6-diaminopyrimidine and their results are shown in Fig. 1. Clews and Cochran conclud that the pyrimidine ring is planar, in agreement with the result of Schneider obtained from dipole moment measurements. The C—N and C—C bonc distances in the pyrimidine ring of these derivatives correspond to approximately 50% double bond character, as would be expected for a six-membered ring in which resonance of the benzene type is possible. Whethe resonance of the benzene type can occur in the amino- and hydroxypyrimidines which are found in nucleic acids is dependent on whether these groups exist in the amino or imino and the enol or keto forms, respectively.

An amino group in position 6(4) of the pyrimidine nucleus may show greatly reduced amino behavior owing to the possibility of a tautomeric change converting the 6-aminopyrimidine (V) into the iminodihydropyrimidine form (VI). The X-ray evidence shows that 4-amino-2,6-dichloropyrimidine and 5-bromo-4,6-diaminopyrimidine[2] are in the amino form


V


VI

in the crystal and it is therefore probable that the structures of cytosin and of 5-methylcytosine are analogous.

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[1] C. J. B. Clews and W. Cochran, *Acta Cryst.* **1,** 4 (1948).

[2] C. J. B. Clews and W. Cochran, *Acta Cryst.* **2,** 46 (1949).

[3] W. C. Schneider, *J. Am. Chem. Soc.* **70,** 627 (1948).

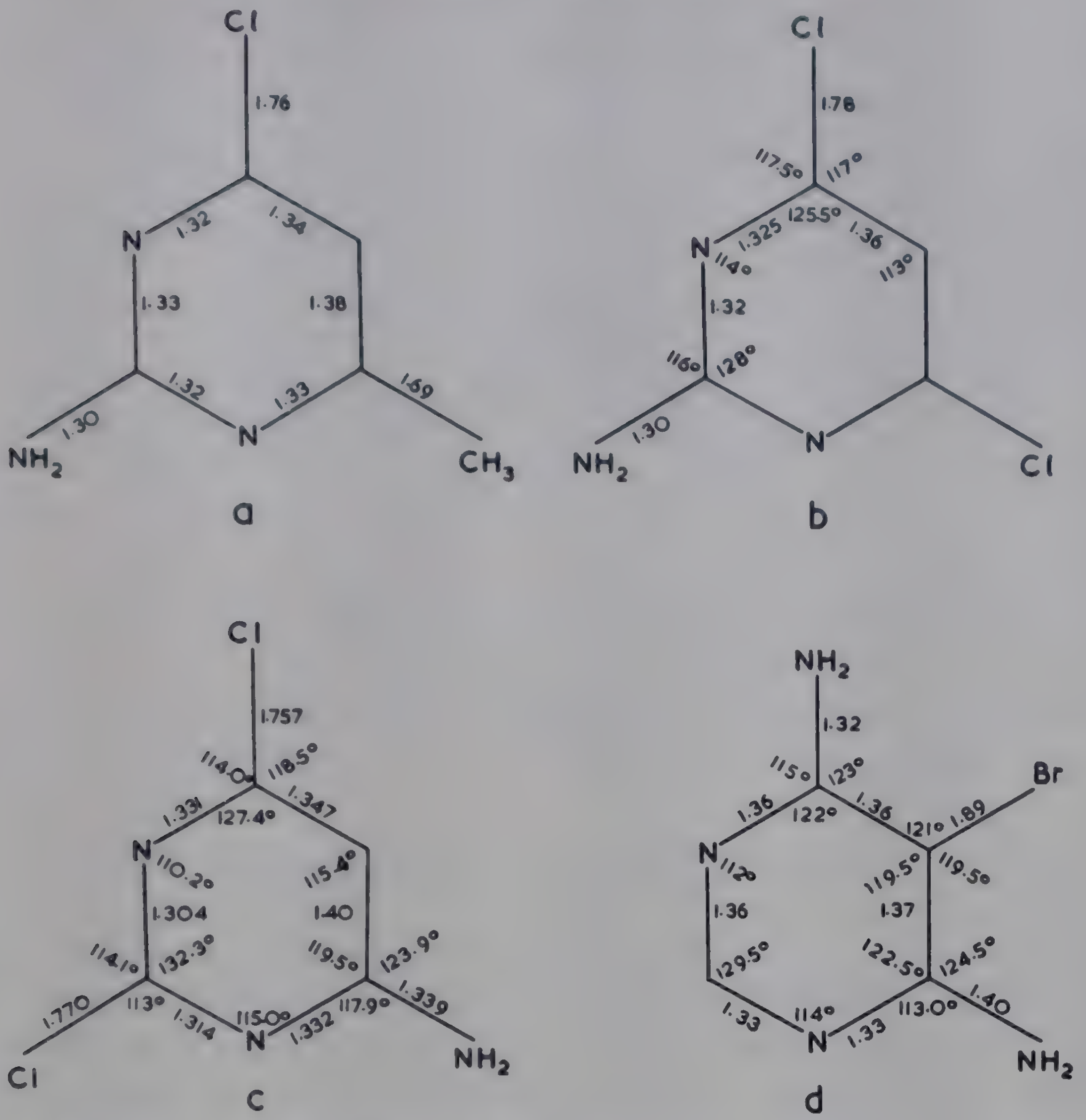

FIG. 1. Structure of (a) 2-amino-4-methyl-6-chloropyrimidine, (b) 2-amino-4,6-dichloropyrimidine, (c) 4-amino-2,6-dichloropyrimidine, and (d) 5-bromo-4,6-diaminopyrimidine (Clews and Cochran[1,2]).

The position of the hydrogen atom in the —NH—CO— groups, i.e., whether the group exists in the keto or enol form, may be determined from the C—O bond length in 2-hydroxy-4,6-dimethylpyrimidine obtained by Pitt,[4] the structure of which is shown in Fig. 2. The length of the C⩵OH bond is 1.25 ± 0.04 A. and is clearly different from the phenolic hydroxyl group in resorcinol (C—OH distance 1.36–1.37 A.) and is to be compared to that in oxalic acid dihydrate (1.24–1.30 A.). The bond between the carbon and oxygen atoms thus possesses considerable double bond character, but the hydrogen atom is nevertheless covalently bound to the oxygen and takes part in a hydrogen bond between the oxygen atom and a water molecule. In aqueous solution, the position of the hydrogen atom is not necessarily the same as in the crystal, and evidence from the ultraviolet absorption spectra of various pyrimidines shows that the keto form is predominant in solution. At room temperatures, in neutral aqueous solution,

[4] G. J. Pitt, *Acta Cryst.* **1**, 168 (1948).

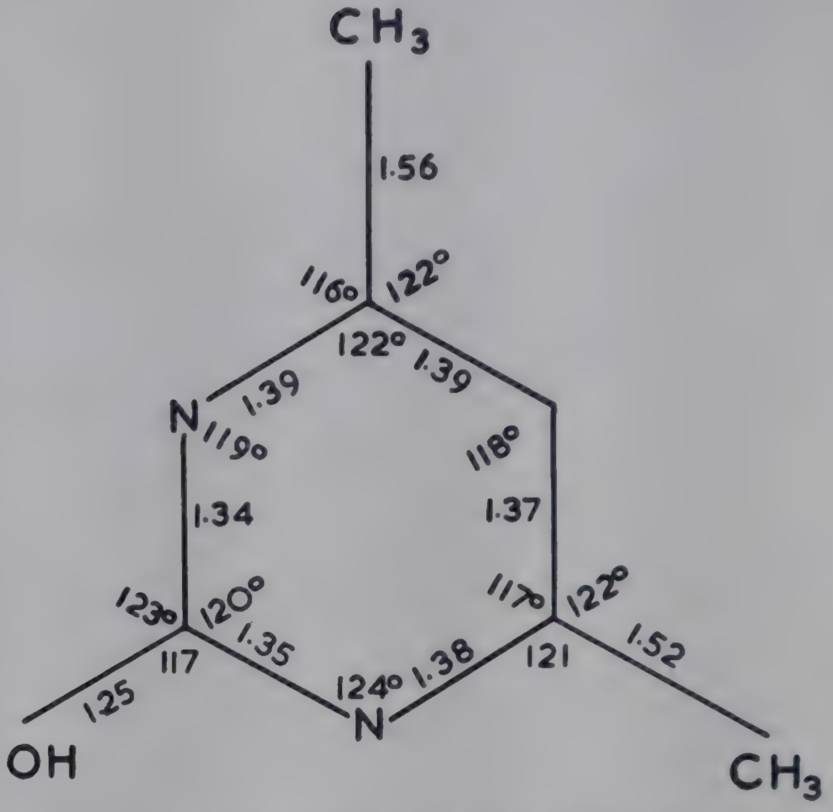

FIG. 2. Structure of 2-hydroxy-4,6-dimethylpyrimidine (Pitt[4]).

the characteristic absorption spectrum of pyrimidines, purines, and of nucleic acids is a broad band around 2400–2800 A. [Cf. *Beaven, Holiday and Johnson*, Chapter 14.]. On increasing the pH of the solution, a shift in $\lambda_{max.}$ to longer wavelengths is observed which has been interpreted by Loofbourow *et al.*[5] and by Stimson and Reuter[6-9] as an enolization of the pyrimidone. However, as Marshall and Walker[10] point out, this shift is more probably due to ionization than to enolization. The problem has been considerably clarified by the work of Marshall and Walker,[10] who have examined the ultraviolet absorption spectra of a number of 2- and 4(6)-substituted pyrimidines and the corresponding N_1- and N_3-methylated derivatives in aqueous solution at various pH values. The pK'_a value of each pyrimidine was determined and the pH of the solution to be measured chosen so as to be $pK'_a \pm 2$. In this way only neutral molecules or ions were present in the solution and the spectra were not confused by being those of mixtures of ions and neutral molecules. Marshall and Walker conclude that the shift in $\lambda_{max.}$ is due to ionization and that potential 2- and 4(6)-hydroxypyrimidines should be represented in the keto form, i.e., as pyrimidones. A similar con-

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[5] J. R. Loofbourow, M. M. Stimson, and M. J. Hart, *J. Am. Chem. Soc.* **65,** 148 (1943).
[6] M. M. Stimson and M. A. Reuter, *J. Am. Chem. Soc.* **65,** 151 (1943).
[7] M. M. Stimson and M. A. Reuter, *J. Am. Chem. Soc.* **67,** 847 (1945).
[8] M. M. Stimson and M. A. Reuter, *J. Am. Chem. Soc.* **67,** 2191 (1945).
[9] M. M. Stimson, *J. Am. Chem. Soc.* **71,** 1470 (1949).
[10] J. R. Marshall and J. Walker, *J. Chem. Soc.* **1951,** 1004.

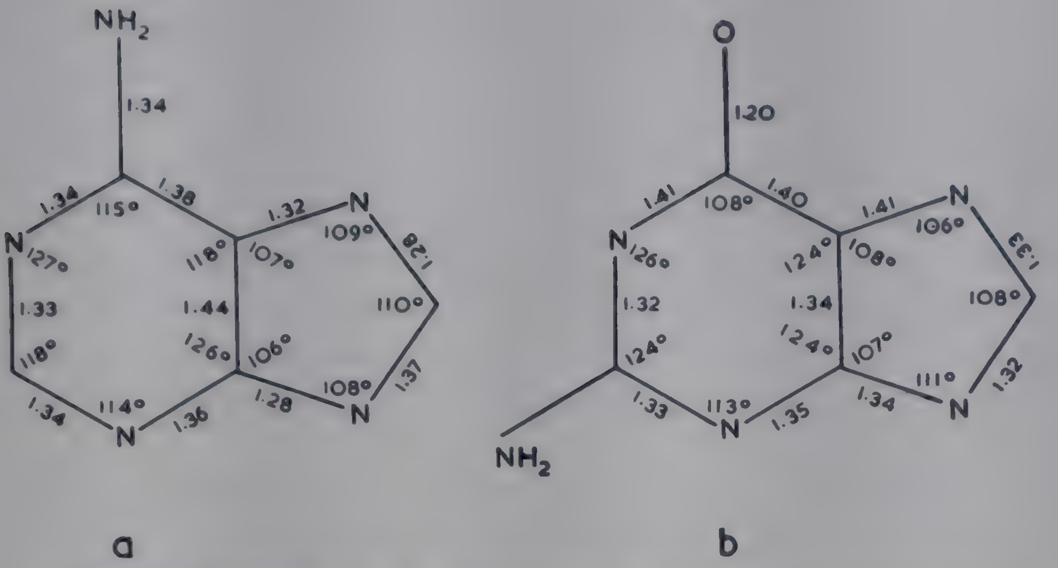
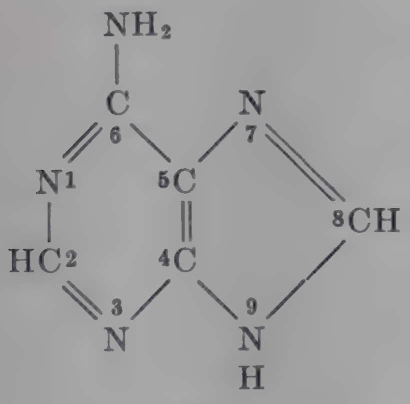


FIG. 3. Structure of (a) adenine, (b) guanine (Broomhead[12,13]).

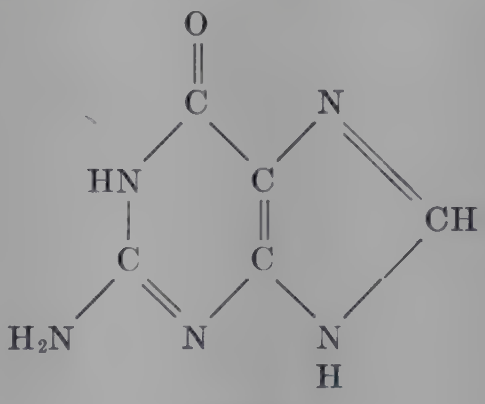
clusion is reached for uracil, which should therefore be represented as 2,4(6)-pyrimidinedione.

b. *Purines*

X-ray studies of adenine hydrochloride were first made by Bernal and Crowfoot,[11] but no attempt was made to determine the positions of the atoms in the unit cell. These have, however, been determined by Broomhead[12,13] for both adenine (VII) and guanine (VIII) hydrochlorides. More precise data on adenine hydrochloride have been obtained by Cochran.[14] The bond distances and interbond angles are given in Fig. 3. The structures of adenine and guanine are very similar, the main differences being that



VII



VIII

the amino nitrogen atom (N_{10}) of guanine appears to be displaced by 0.11 A. from the plane containing the other atoms and that there is a difference of

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[11] J. D. Bernal and D. Crowfoot, *Nature* **131,** 911 (1934).

[12] J. M. Broomhead, *Acta Cryst.* **1,** 324 (1948).

[13] J. M. Broomhead, *Acta Cryst.* **4,** 92 (1951).

[14] W. Cochran, *Acta Cryst.* **4,** 81 (1951).

0.10 A. in the C_4—C_5 bond distance, that in adenine being the longer. This difference may be due to experimental error,[13] but nevertheless is in agreement with the different acid-base properties of adenine and guanine (see p. 457). The C_6—O bond in guanine, which has a length of 1.20 A. appears to have predominantly double bond character, thus confirming the conventional keto formula (VIII) ascribed to guanine. Broomhead[13] points out, however, that an error of only -0.05 A. in this value (i.e., giving a bond length of 1.25 A.) would make the bond of comparable length to that of the C—O bond in glycine and diketopiperazine, where the bond possesses only 50% double bond character.

The position of the hydrogen atoms has been considered by both Broomhead[13] and Cochran,[14] who conclude that in the adenine cation, hydrogen atoms are at N_1, C_2, C_8, N_9, and two hydrogen atoms are at the amino nitrogen N_{10}. The positive charge may be located at N_1, N_9, or N_{10} and the short length of the C_6—N_{10} bond (1.34 A. compared with the single C—N bond length of 1.47 A.) may be attributed to the contribution of resonance forms in which the N_{10} bears a positive charge and in which the C_6—N_{10} bond is double. The location of the hydrogen atoms in the guanine cation is not known so precisely. Four hydrogen atoms are located at N_1, N_9, N_{10}, and C_8, and hydrogen bonds exist between N_3 and N_{10} and between O and N_7. There are thus four possible tautomeric forms, and a decision as to which is the correct structure must await the results of a more precise study. In aqueous solution, it will be difficult to distinguish between the four "mesohydric tautomers" and indeed such distinction may be meaningless.

c. *Nucleosides*

The structure of only one nucleoside, viz., cytidine (IX), has been determined in detail although some preliminary studies have been made on the other ribonucleosides. The deoxypentose nucleotides have yet to be examined. The structure of cytidine has been determined by Furberg[15,16] and in addition to confirming the furanose structure of the D-ribose, the point of attachment of the sugar radical as being at N_3, and the β-configuration of the N_3-glycosidic link, other information of fundamental importance to the structure of nucleic acids has emerged. The molecular projection is given in Fig. 4. The six atoms of the pyrimidine ring lie in the same plane in agreement with the observations of Clews and Cochran[1,2] and of Pitt[4] on some substituted pyrimidines. The C_2—O distance (see IX) is 1.25 A., identical with the value found by Pitt[4] for the corresponding bond in 2-hydroxy-4,6-dimethylpyrimidine, and the bond therefore possesses some double bond character. The bond from the ring to the amino group,

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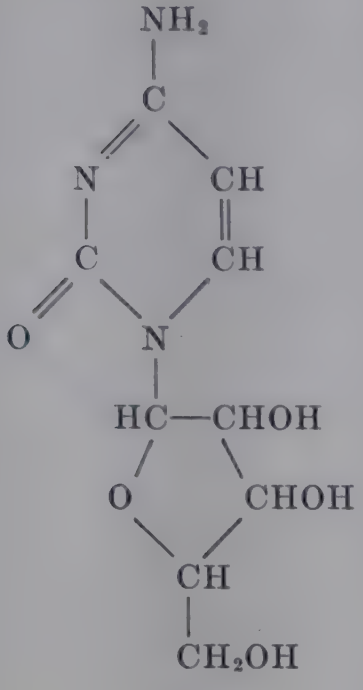
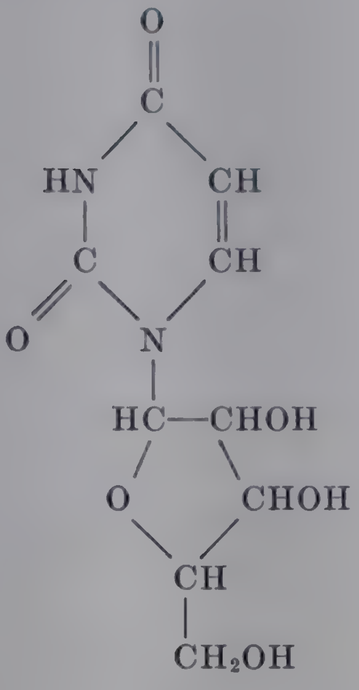
[15] S. Furberg, *Nature* **164,** 22 (1949).

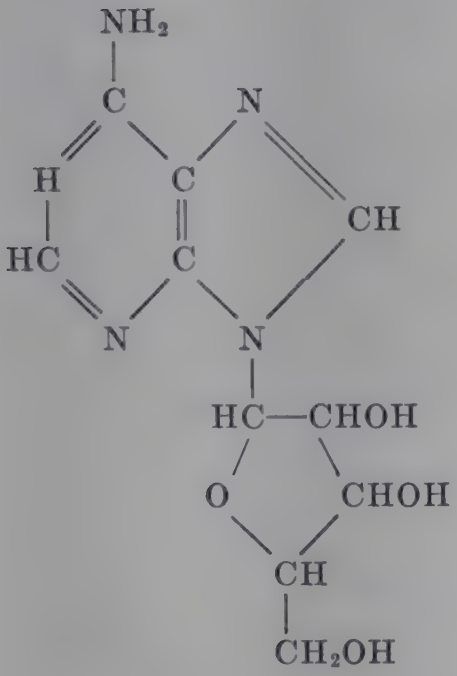
[16] S. Furberg, *Acta Cryst.* **3,** 325 (1950).



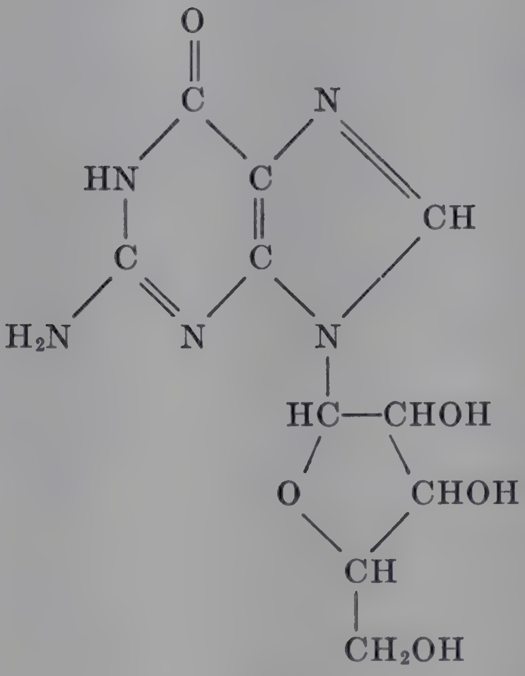
IX



X



XI



XII

C_6—N, is short (1.31 A.), suggesting that it participates in the resonance of the pyrimidine ring. Four of the atoms of the D-ribose ring lie nearly in one plane, viz., C_1', O_1', C_2', and C_4', but the fifth atom of the ring, C_3', is out of the plane by nearly 0.5 A. The central bond, N_3—C_1, joining the pyrimidine ring to the sugar radical has a length of 1.47 A. and is clearly a single bond. This bond lies in the plane of the pyrimidine ring and forms angles of 109° and 115° with the adjacent ring bonds in D-ribose. Contrary to the previous assumption that the two rings were parallel [see Astbury[17]], it is evident that they are approximately perpendicular. The bearing of this

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[17] W. T. Astbury, *Symposia Soc. Exptl. Biol.* **1,** 66 (1947).

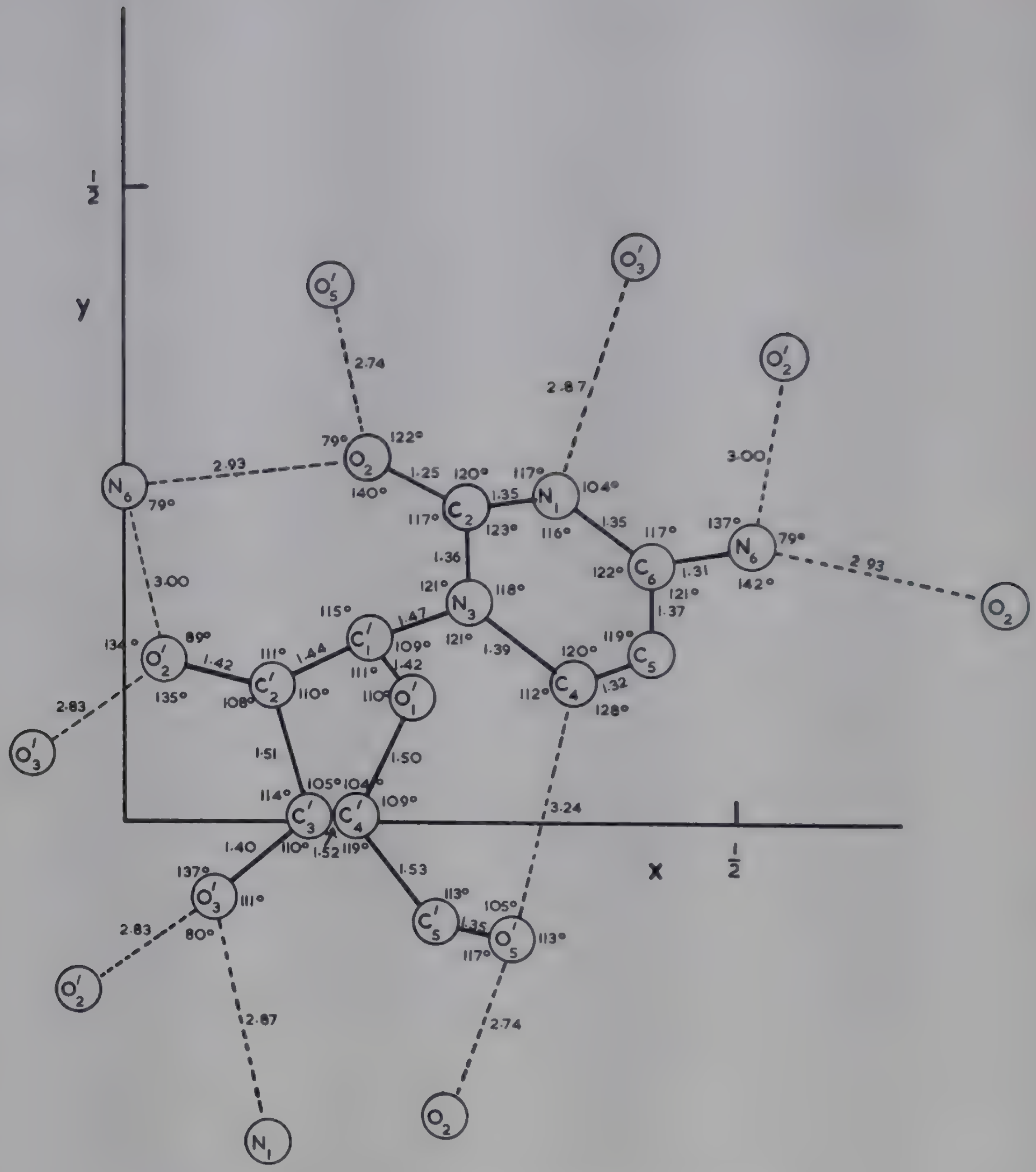

FIG. 4. Dimensions of cytidine (Furberg[15,16])

important result on the structure of nucleic acid will be discussed below (see p. 462).

Preliminary X-ray investigations have also been carried out by Furberg on uridine (X), adenosine (XI), and guanosine (XII).[18] From the resemblance of the cell dimensions of uridine to those of cytidine, it is concluded that the stereochemistry of the two molecules are very similar. The data on adenosine confirm that the glycosidic linkage is at N_9 and is of the β-type. The direction of the N_9—C'_1 bond connecting the two rings was considered by Hendricks[19] and Astbury[17] to form an angle with the plane of the purine ring so as to make the purine and D-ribose rings parallel. As

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[18] S. Furberg, *Acta Chem. Scand.* **4,** 751 (1950).

[19] S. B. Hendricks, *J. Phys. Chem.* **45,** 65 (1941).

Furberg has shown in cytidine, the corresponding bond lies in, or very nearly in, the plane of the pyrimidine ring, and the considerable resonance in the adenine molecule would suggest that this is true for adenosine. If so, the two rings will be nearly perpendicular to each other, as in cytidine. Although the existing data do not definitely exclude other configurations, this structure appears to give the most satisfactory explanation of the available information. The data of Hendricks[19] can be reinterpreted on the basis of this model. Gulland *et al.*[20] have pointed out that, in the structure proposed by Hendricks, the sugar was α-lyxose and not D-ribose; and, in the structure proposed by Furberg, two parallel planes approximately 1.5 A. apart can be recognized, one containing the purine ring and C_1', C_4', and C_5', the other passing through C_2', C_3', and O_2'.[18] Furberg[18] considers that guanosine has a structure similar to adenosine.

2. Dissociation Constants

a. Pyrimidines and Purines

The pK'_a values of some pyrimidines and purines are given in Table I. The acid-base properties of uracil were first investigated by Levene *et al.*,[21,22] who concluded from electrometric titration data that it possessed two acid dissociations with pK'_a values 9.28 and 13.56, which were attributed to the two —CO—NH— groups. Later the same authors[23] showed that an error had been made in applying the water correction for the titration in strongly alkaline solution and a recalculation of the data showed the presence of only a single dissociation of pK'_a 9.45. This conclusion has been confirmed by Taylor.[24] The reason for the extreme weakness of the second dissociation constant of uracil is still obscure. Levene *et al.*[23] considered that in the neutral molecule the two —CO—NH— groups were of comparable strength, a view that is supported by the pK'_a values of 1- and 3-methyluracil which are similar to that of uracil (Table I), and that the ionization of the second group was inhibited by the ionization of the first to such an extent that its pK'_a was too high to be detected by titration in aqueous solution. However, calculation of the difference between pK'_{a_1} and pK'_{a_2},[25] using the formulas of Kirkwood and Westheimer[26,27] and assuming that the dimensions of the uracil molecule are similar to those of 2-hydroxy-4,6-dimethylpyrimidine,[4] gives the low value of 1.57. It would therefore appear that the weakness of the second dissociation cannot be due to the electrostatic field effect and must be caused by a real difference in the groups. This conclusion is not entirely unexpected, since N_1H lies between two carbonyl groups, whereas N_2H is attached to a carbonyl group and a CH group. This view is also supported by the extreme weakness of the second dissociation of cytosine, pK'_{a_2} 12.1, as compared with that of isocytosine (XIII), pK'_{a_2} 9.42, which suggests that the 2-carbonyl group in uracil promotes stronger basic properties in the—NH—CO—grouping than does the 6-carbonyl group. This conclusion is similar to that reached by Ogston[28] in the somewhat analogous case of the xanthines.

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[20] J. M. Gulland, G. R. Barker, and D. O. Jordan, *Nature* **151,** 109 (1943).

[21] P. A. Levene and H. S. Simms, *J. Biol. Chem.* **65,** 519 (1925).

[22] P. A. Levene, H. S. Simms, and L. W. Bass, *J. Biol. Chem.* **70,** 243 (1925).

[23] P. A. Levene, L. W. Bass, and H. S. Simms, *J. Biol. Chem.* **70,** 229 (1926).

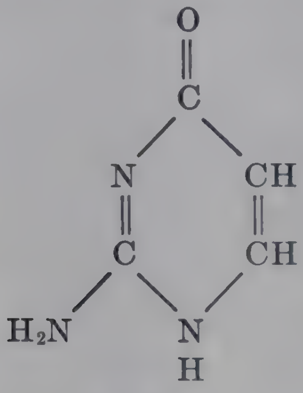
[24] H. F. W. Taylor, Acid Base Properties of Nucleic Acids, Doctoral Thesis, London Univ., London, England, 1946.

[25] D. O. Jordan, *Progr. Biophys. and Biophys. Chem.* **2,** 51 (1951).

[26] J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.* **6,** 506 (1938).

[27] F. H. Westheimer and J. G. Kirkwood, *J. Chem. Phys.* **6,** 513 (1938).

The introduction of the methyl group in the 5-position of uracil in thymine has little effect on the pK'_a value. The basic properties of cytosine and isocytosine can be ascribed to the amino group, the pK'_a values being



XIII

comparable with those of the aromatic amines; this view has been confirmed by titration in the presence of formaldehyde.[24]

The dissociation constants of adenine and guanine are given in Table I. There is considerable evidence that the weakest dissociation of adenine and guanine represents the dissociation —NH— to $—\bar{N}—$ in the imidazole ring. Benzimidazole, which is analogous to purine, has been found by Taylor[29] to have a basic association of pK'_{a_1} 5.30 and an acidic dissociation of pK'_{a_2} 12.3. These values must represent the dissociation of the $—\overset{+}{N}H=$ and —NH— groups, respectively. The value of pK'_{a_1} 5.30 may be compared with that of 7.1 given by Dedichen[30] for imidazole, whereas the value of 12.3 is of the same order as those of the weakest dissociations of guanine, 1- and 3-methylxanthine, and of hypoxanthine, all of which are unsubstituted in the imidazole ring. As would be expected, 7- and 9-methyl-

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[28] A. G. Ogston, *J. Chem. Soc.* **1935,** 1376.

[29] H. F. W. Taylor, *J. Chem. Soc.* **1948,** 765.

[30] G. Dedichen, *Ber.* **39,** 1831 (1906).

TABLE I

pK'_a VALUES OF PYRIMIDINES AND PURINES

	pK'_{a_1}	pK'_{a_2}	pK'_{a_3}	Reference
Uracil	9.45			21–24
1-Methyluracil	9.99			23
3-Methyluracil	9.71			23
Thymine	9.82			23
Cytosine	4.60	12.16		23,24
Isocytosine	4.01	9.42		23
Adenine	4.15	9.80		29
Guanine	3.3	9.20	12.3	29
Xanthine	7.7			28
1-Methylxanthine	7.7	12.05		28,29
3-Methylxanthine	8.5,[a] 8.10[b]	11.3		28,29
7-Methylxanthine	8.5,[a] 8.30[b]			28,29
9-Methylxanthine	6.3,[a] 6.25[b]			28,29
Hypoxanthine	8.8	12.0		24

[a] Results of Ogston.[28]

[b] Results of Taylor.[29]

xanthine show no dissociation in the pH range 11.0–12.5. In adenine, the pK'_{a_2} value, 9.80, is surprisingly low when compared with the corresponding dissociation in guanine and has been explained by Taylor[31] on the basis of the crystallographic data of Broomhead.[12,13] Taylor explains the unusual acid strength of this group in adenine as being due to the considerable contribution to the resonance of the structure, not only of the uncharged structures, but also of those in which there is a negative charge on the nitrogen atoms in the pyrimidine ring and a positive charge on those in the imidazole ring. This distribution of charge would lower the pK'_{a_2} value from that characteristic of an uncharged —NH— group towards one characteristic of a charged $—\overset{+}{\mathrm{N}}\mathrm{H}=$ group. The respective pK'_a values for these groups in benzimidazole are 12.3 and 5.3 and the observed value of 9.8 for adenine may thus be explained. In guanine, the dissociation of the imidazole —NH— group takes place in a molecule which already bears a negative charge in the region of the pyrimidine nitrogen atoms as a result of the dissociation of the —NH—CO— group. It is therefore to be expected that the resonance structures bearing a negative charge on the negative atoms of the pyrimidine ring will make a smaller contribution in the guanine than in the adenine anion, and, since resonance structures of this type necessarily contain a single bond in the C_4—C_5 position, Taylor[31] concludes that the greater length of this bond in adenine is explained. This argument has been criticized by Cochran[14] on the grounds that the resonance structures of the type considered by Taylor consistently make the bonds N$_9$—C and N$_7$—C$_5$ single bonds and the bond N$_7$—C$_8$ a double bond, whereas th X-ray data indicate that these bonds in adenine all possess 20–50% doubl bond character. However, the correlation of bond lengths obtained for th crystal with other properties obtained from measurements made on solutions must be carried out with care as the different environments ma produce small changes in the molecule. In the present instance, the influenc of the chloride ion, which exists close to the adenine ion in the crystal lattice, may produce significant effects.

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[31] H. F. W. Taylor, *Nature* **164**, 750 (1949)

The group responsible for the acid dissociations of pK'_a 6–9 which appear in the oxypurines is not known with certainty. Existing evidence indicates clearly that it is to be associated with the presence of the 6-oxy group in guanine, hypoxanthine and xanthine; the second oxy group in the 2-position of xanthine does not give rise to a further dissociation, the case being somewhat analogous to that of uracil. Ogston[28] considers that the acidic properties of xanthine do not depend on the —NH—CO— group in the 1- and 2-positions since substitution by a methyl group in the 1-position does not greatly alter the pK'_{a1} value of xanthine, nor that of the 3-, 7-, and 9-methylxanthines. The actual form of the dissociating groups has been studied by Ogston[28] by electrometric titration in water and 90% ethyl alcohol. In xanthine, the group is considered to be in the enolic form and the structure is —N$_1$=C$_6$OH. In the 3-, 7-, and 9-methylxanthines, however, the zwitterionic form is considered to predominate, the acid dissociation being represented as that of an—$\overset{+}{\mathrm{N}}$H=group in the imidazole ring. If this interpretation is correct, the second dissociation in the imidazole ring must be preceded, according to Taylor,[29] by a tautomeric change, since Ogston[28] has shown that the —NH— group in the 1-position does not show acid properties in these molecules.

b. Nucleosides and Nucleotides

The pK'_a values of the ribonucleosides and ribonucleotides are given in Table II. The values for the nucleosides correspond closely to those of the parent purines and pyrimidines although there is a general tendency for the pK'_a values to be lowered, i.e., for the acid dissociations to be strengthened. Except in the case of xanthosine, which has not been studied to a sufficiently high pH value, an additional dissociation is observed at pK'_a 12.3–12.6, which is of the correct order for the first acid dissociation of a sugar. No data are available for D-ribose, but glucose has a pK'_a value of 12.1.[32-34]

The pK'_a values of the ribonucleotides obtained from yeast nucleic acid are given in Table II. These values will all refer to mixtures of the *a* and *b* nucleotides, i.e., of the nucleoside-2′- and -3′-phosphates, in unknown ratios. This isomerism, however, will have only a small effect on the dissociation constants (see below). Comparison of the values for the nucleo-

[32] P. Hirsch and R. Schlags, *Z. physik. Chem.* **141**, 387 (1929).

[33] F. Urban and P. A. Shaffer, *J. Biol. Chem.* **94**, 697 (1931).

[34] F. Urban and R. D. Williams, *J. Biol. Chem.* **100**, 237 (1933).

TABLE II

pK'_a VALUES OF NUCLEOSIDES AND NUCLEOTIDES

	pK'_{a_1}	pK'_{a_2}	pK'_{a_3}	pK'_{a_4}	Reference
Adenosine	3.5	12.5			21,22
Guanosine	1.6	9.2	12.3		21,22
Cytidine	4.2	12.3			21,22
Uridine	9.2	12.5			21,22,35
Inosine	8.8	12.3			21,22
Xanthosine	6.0				28
Adenylic acid	0.9	3.7	6.0		21,22,35
Guanylic acid	0.7	2.4	6.0	9.3	21,22,35
Cytidylic acid	0.8	4.2	6.0		21,22,35
Uridylic acid	1.0	5.9	9.4		21

tides with those of the respective nucleosides, shows that the dissociating groups of the latter are supplemented by two additional dissociations having pK'_a values 0.7–1.0 and 5.9–6.0. These are of the correct order for the first and second dissociations of a sugar phosphate; ribose phosphates have not been studied, the analogous glucose-3-phosphate has pK'_a values of 0.84 and 5.67,[36] and similar values have been obtained for other sugar phosphates.[37]

The assignment of the pK'_a values to the various groups is best carried out by reference to the data for the corresponding nucleosides and sugar phosphates. The following are the relevant data for adenylic acid:

Adenosine	pK'_a 3.5		
Sugar phosphates	pK'_{a_1} 0.8–1.1	pK'_{a_2} 6.0–6.5	
Adenylic acid	pK'_{a_1} 0.9	pK'_{a_2} 3.7	pK'_{a_3} 6.0

The pK'_{a_1} value of adenylic acid is clearly that of a primary phosphoric acid dissociation, pK'_{a_2} an amino dissociation, and pK'_{a_3} a secondary phosphoric acid dissociation. In the isoelectric region, therefore, it is evident that adenylic acid, and hence also guanylic and cytidylic acids, will exist largely in the zwitterionic form. It is therefore to be expected that the nucleic acids will behave similarly.

The values of K_z for the nucleotides, where

$$K_z = \frac{[H_3\overset{+}{N}ROPO(OH)\bar{O}]}{[H_2NROPO(OH)_2]}$$

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[35] W. E. Fletcher, On the Structure of Nucleic Acids, Doctoral thesis, London Univ., London, England, 1948.

[36] O. Meyerhof and K. Lohmann, *Biochem. Z.* **185,** 113 (1927).

TABLE III

VALUES OF K_z FOR THE AMPHOLYTIC NUCLEOTIDES

	pK'_{a_1} (nucleotide)	pK'_{a_1} (nucleoside)	K_z
Guanylic acid	0.7	1.6	7
Adenylic acid	0.9	3.5	3.6×10^2
Cytidylic acid	0.8	4.2	2.6×10^3

have been calculated[25] assuming (1) that only the primary phosphoric acid grou and the amino group are important in determining the position of the isoelectri region and the value of K_z , (2) that the constant for the dissociation of the nucleotid

$$H_3\overset{+}{N}ROPO(OH)_2 + H_2O \rightleftharpoons H_2NROPO(OH)_2 + H_3O^+$$

is identical with that for the nucleoside

$$H_3\overset{+}{N}R + H_2O \rightleftharpoons H_2NR + H_3O^+$$

Then[38]

$$K_z = \frac{K_1(\text{nucleotide})}{K_1(\text{nucleoside})} - 1$$

This assumption is analogous to that generally made for calculating K_z for the amin acids, when it is assumed that the dissociation of the amino group in the positivel charged acid is identical with that of the corresponding ester.[38] It is justified her by the fact that the undissociated phosphoric acid group is unlikely to have an inductive effect on the amino group, from which it is separated by six atoms, an the furanose ring is a nonresonating system. The values of K_z so calculated are give in Table III. In guanylic acid, because of the low value of the pK'_a of the amino grou in guanosine, the concentration of the zwitterionic form is only seven times that o the uncharged form. In adenylic and cytidylic acids, however, the ratio is very muc greater and the concentration of the uncharged form in the isoelectric region i negligible.

It is evident from Table II that the introduction of the negatively charge phosphate ion into the nucleoside produces a weakening of the acid strengt of the amino and the —NH—CO— dissociations. This observation is i agreement with the field effect, first recognized by Bjerrum[39] and treate quantitatively by Kirkwood and Westheimer.[26,27] The degree of the shif of the pK'_a will be a function of the distance between the charged groups the smaller this distance the greater the pK'_a shift. This relationship ha been used by Cavalieri[40] in an attempt to establish the configuration of th

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[37] W. D. Kumler and J. J. Eiler, *J. Am. Chem. Soc.* **65**, 2355 (1943).

[38] E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides." Chemica Catalog Co., New York, 1943.

[39] N. Bjerrum, *Z. physik. Chem.* **106**, 219 (1923).

[40] L. F. Cavalieri, *J. Am. Chem. Soc.* **74**, 5804 (1952).

a and *b* isomers of cytidylic acid. From electrometric titration (glass electrode) the following pK'_a values were obtained:

a	pK'_{a_2} 4.36 (4.3)	pK'_{a_3} 6.17 (6.2)
b	pK'_{a_2} 4.28 (4.3)	pK'_{a_3} 6.0 (6.0)

The values in parenthesis were those obtained by Loring *et al.*,[41] and Cavalieri considers that since the amino and phosphate groups will be closer in the 2′- than in the 3′-phosphate the *a* isomer is probably the 2′-phosphate. A similar conclusion was reached by Loring *et al.*[42] on the grounds that the isomer with the smaller distance between the charged groups would show the greater tendency towards zwitterion formation and hence have the lower solubility, acidity, and ultraviolet absorption.

II. Nucleic Acids

1. Structure Determinations by X-Ray Diffraction

Although the general chemical picture of the nucleotides and of the nucleic acids is one of some complexity, the basic macromolecular configuration which emerges from X-ray diffraction studies is one of comparative simplicity. The studies, in the main, have been concerned with the sodium salt of deoxypentose nucleic acid since this substance can be comparatively readily isolated in a state of purity and of high molecular weight. Ribonucleic acids have only been examined when a high-molecular-weight sample, such as that obtained from tobacco mosaic virus, has been available.

The main features of the molecular structure of deoxypentose nucleic acid have been known since 1938, but it is only recently that the accumulation of knowledge has permitted the suggestion of a structure concerning which there appears to be a large measure of agreement. Astbury and Bell[43] showed first that the X-ray fiber photograph gave a prominent reflection which corresponded to a spacing of 3.34 A. along the fiber axis. This was interpreted as due to a succession of flat nucleotides standing out perpendicularly to the fiber axis to form a relatively rigid structure. This rather small distance between the nucleotides is in agreement with the density of the dried nucleic acid, which is 1.62–1.63 g./cc. In addition to this spacing of the nucleotides, Astbury and Bell and later Astbury[17] concluded that the structure pattern along the axis of the molecule repeated at a distance of about 27 A. Since the Astbury structure assumed a pile of nucleotides, one on top of the other, this distance corresponds to approximately eight nucleotides. In view of the more recent developments it shoul be pointed out that Astbury[17] did not completely reject the possibility tha the nucleotides might be disposed spiralwise around the long axis of th molecule, but he concluded that if the nucleotides did not lie closely on to of one another, then the state of packing must be dimensionally equivalen to such an arrangement so that the neighboring nucleotide molecules wer closely interleaved in a surprisingly regular pattern. This, Astbury considered, was unlikely.

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[41] H. S. Loring, H. W. Bortner, L. W. Levy, and M. L. Hammell, *J. Biol. Chem.* **196,** 807 (1952).

[42] H. S. Loring, M. L. Hammell, L. W. Levy, and H. W. Bortner, *J. Biol. Chem.* **196,** 821 (1952).

[43] W. T. Astbury and F. O. Bell, *Nature* **141,** 747 (1938).

The Astbury structure rested, in part, on the assumption that the nucleotides were flat or approximately flat molecules, i.e., the purine or pyrimidine ring systems were in the same plane as that of the sugar. This wa shown to be wrong by Furberg,[15,16] who showed that in the nucleosid cytidine the two ring systems instead of being parallel were almost perpendicular. This observation is of fundamental importance in the development of the structure of nucleic acids and has been confirmed fo 2′,3′-isopropylidene-3,5′-cycloadenosine iodide by Clark *et al.*[44] and by Zussman.[44a] As has been pointed out above, the bond between sugar and purin or pyrimidine ($N_{9\text{ or }3}$—C_1') is a single bond and so rotation about thi bond is possible. However, Furberg[45] finds that not all orientations of th sugar and purine or pyrimidine are equally feasible and the most favorabl position is considered that shown in Fig. 5 which is that found in the crysta structure of cytidine.[15,16] In view of these observations on the structure o the nucleosides and nucleotides, Furberg[45] revised the Astbury structure and proposed two possible models which are given in Fig. 6. In contrast t the Astbury model, these two modifications have the planes of the suga rings, as well as the P—O_3' bonds (Fig. 5), approximately parallel to th long axis of the molecule. Most atoms, including the phosphorus atoms lie in planes 3.4 A. apart, thus explaining the strong 3.4-A. reflection.

In model 1 (Fig. 6) the pyrimidine and purine rings are piled almost on top of eac other; they cannot be piled directly on top of each other without bringing some o the atoms in successive nucleotides too near together. Van der Waals attraction between the rings will stabilize the structure. The ribose rings and the phosphate group form a spiral enclosing the column, the spiral repeating itself after eight nucleotide as required by the Astbury model. In model 2, the ribose rings and phosphate group form a central column from which the purines and pyrimidines stand out perpendicularly. This model has the disadvantage that there are no intramolecular Van de Waals forces between the purines and pyrimidines, but the rigidity of the molecul will depend on such forces between the sugar molecules.

Following the successful formulation of the structure of some of the pro

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[44] V. M. Clark, A. R. Todd, and J. Zussman, *J. Chem. Soc.* **1951,** 2952.

[44a] J. Zussman, *Acta Cryst.* **6,** 504 (1953).

[45] S. Furberg, *Acta Chem. Scand.* **6,** 634 (1952).

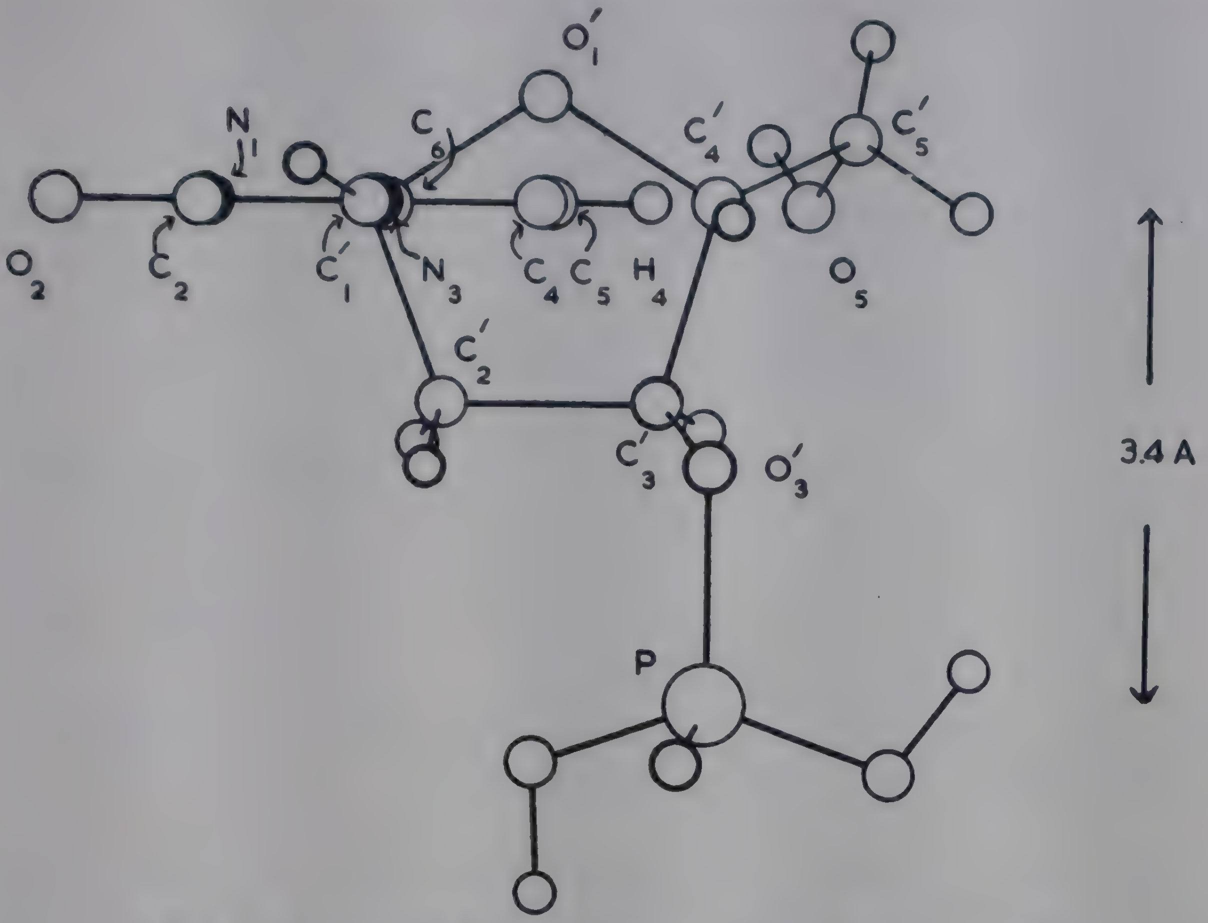

FIG. 5. Structure of a pyrimidine nucleoside (Furberg[45]).

teins as helical polypeptide chains, Pauling and Corey[46,47] suggested a structure for nucleic acid which they considered as compatible with the main features of the X-ray diagram. The structure involved three intertwined helical polynucleotide chains, each chain having approximately twenty-four nucleotides in seven turns of the helix. This structure, however, has been severely criticized on several grounds. First, the Pauling and Corey structure has the phosphate groups closely packed about the axis of the molecule, with the pentose residues surrounding them and the purine and pyrimidine groups projecting radially; the general behavior of the nucleate ion in solution, however, would suggest the reverse configuration, i.e., with the phosphate groups on the outside of the molecule and the purine and pyrimidine rings on the inside. There is considerable experimental evidence for this, the most important probably being that of Gulland *et al.*,[48] who showed by titration that the phosphate groups are available for acid-base equilibria in the normal way and that the amino and the —NH—CO— groups on the purines and pyrimidines were inaccessible, unless the nucleic acid was first subject to extremes of pH. Dye and protein absorption confirm that the phosphate groups must be accessible to large ions. Secondly,

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[46] L. Pauling and R. B. Corey, *Nature* **171,** 346 (1953).

[47] L. Pauling and R. B. Corey, *Proc. Natl. Acad. Sci. U. S.* **39,** 84 (1953).

[48] J. M. Gulland, D. O. Jordan, and H. F. W. Taylor, *J. Chem. Soc.* **1947,** 1131.

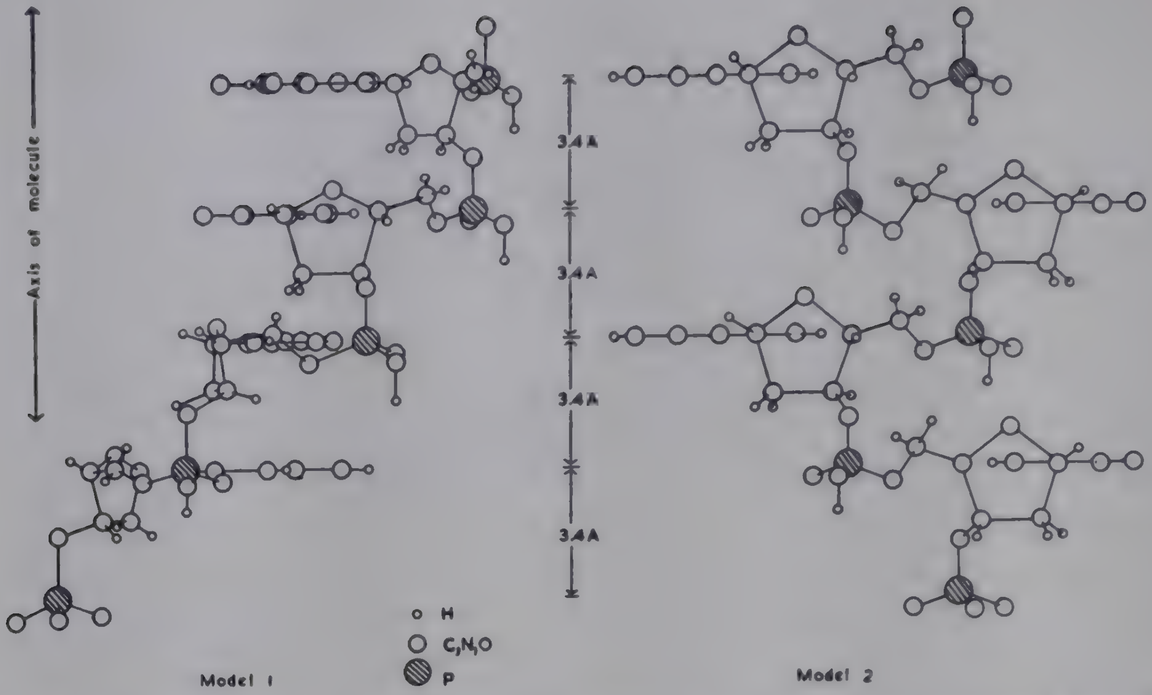

FIG. 6. Two models of deoxypentose nucleic acid based on nucleotides of the standard configuration (see Fig. 5) (Furberg[45]).

Watson and Crick[49] point out that many of the Van der Waals distances in the Pauling and Corey structure are too small and also that while the X-ray diagrams are for the sodium salt of deoxypentose nucleic acid, the Pauling and Corey structure refers to the free acid. The great importance of the Pauling and Corey contribution probably lies in the suggestion that the structures of nucleic acids were of the helical type.

In view of these criticisms a new structure was proposed by Watson and Crick[49] which has many novel features and appears to be in harmony with a large amount of different experimental data, and it would appear, apart from alterations of detail, that this structure is correct for the deoxyribonucleate ion as obtained from calf thymus. This structure has two helical chains each coiled round the same axis (see Fig. 7). Both chains follow right-handed helices, but the sequences of the atoms in the two chains run in opposite directions. Each chain resembles the Furberg[45] formula model 1 (Fig. 6) and the configuration of the nucleotides are approximate to that given in Fig. 5. The pyrimidines and purines are thus on the inside of the helix and the phosphates on the outside, and a nucleotide occurs in the direction of the long axis every 3.4 A. The structure repeats every ten nucleotides or 34 A., the angle between adjacent nucleotides in the same chain being assumed to be 36°.

The novel feature of the structure is the manner in which the two chains are held together by the purine and pyrimidine bases. The planes of these bases are perpendicular to the fiber axis and are joined together in pairs by

[49] J. D. Watson and F. H. C. Crick, *Nature* **171,** 737 (1953).

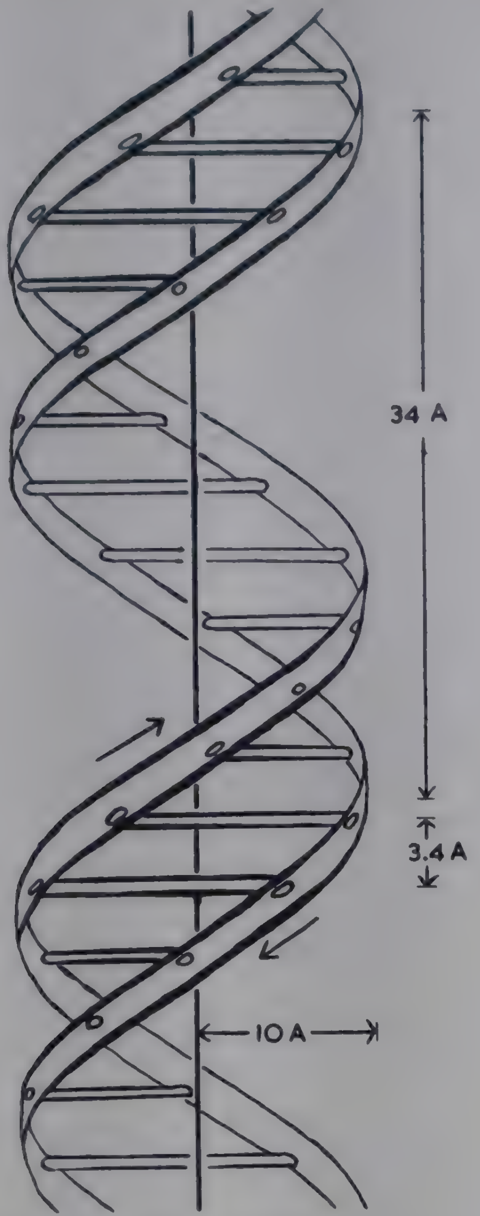

Fig. 7. The helical structure of sodium deoxypentose nucleate proposed by Watson and Crick.[49]

hydrogen bonds. A single base from one chain is hydrogen-bonded to a single base in the other chain so that the two bases lie side by side with identical z-coordinates. The formation of these hydrogen bonds between different bases is found, on the basis of molecular models, to be highly specific and only certain pairs of bases will fit into the structure. One member of a pair must be a purine and the other a pyrimidine in order to bridge between the two chains. A bridge of two pyrimidines is not large enough to form the link and there is not room for two purines. If the most probable tautomeric forms of the purines and pyrimidines are assumed it is then found that the only pairs of bases that are possible are:

adenine with thymine
guanine with cytosine

The ways in which these can be joined are shown in Fig. 8. A given pair can

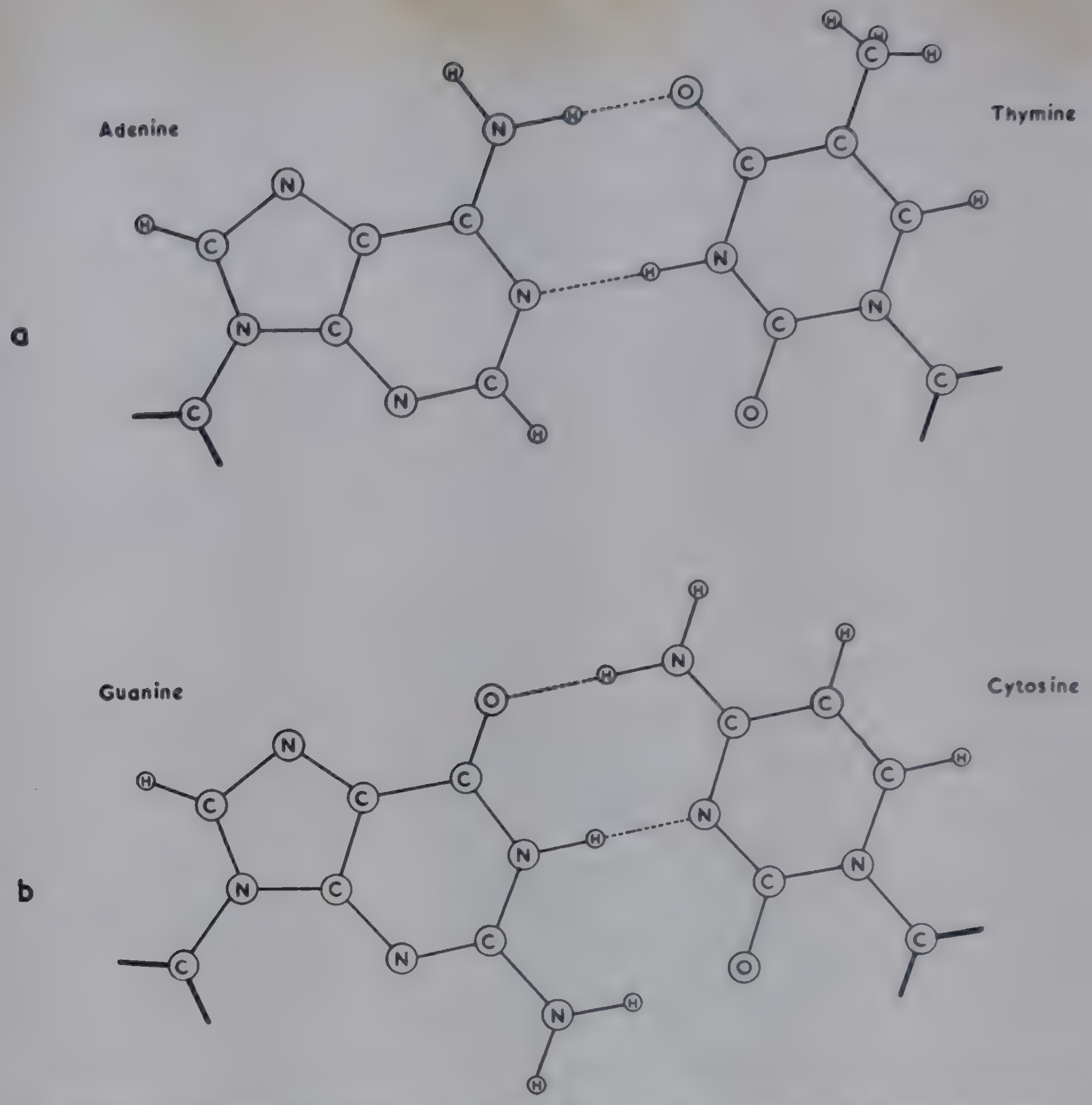

FIG. 8. The permitted hydrogen bonds in the Watson and Crick formula.

be either way around, thus adenine can occur in either chain, but when i does, its partner on the other chain must always be thymine.

The pairing of the bases in this manner is strongly supported by th recent analytical results of Chargaff[50] and Wyatt.[51,52] The molar ratios o adenine–thymine and guanine–cytosine for a variety of deoxypentos nucleic acids are listed in Chapter 10. These ratios approximate very closel to unity in accordance with the Watson and Crick hypothesis. The ratio of adenine to guanine on the other hand [see Chapter 10] are generall much greater than unity and are variable in different nucleic acids. Th formation of hydrogen bonds between the paired bases, as shown in Fig 8, is also in agreement with the titration results of Gulland *et al.*,[48] who firs suggested the formation of hydrogen bonds between the amino and —NH— CO— dissociations in order to explain the nonavailability of this grou

[50] E. Chargaff, *Experientia* **6,** 201 (1950); *Federation Proc.* **10,** 654 (1951); for oth references see Chapter 10 of this book.

[51] G. R. Wyatt, *Biochem. J.* **48,** 584 (1951).

[52] G. R. Wyatt, *J. Gen. Physiol.* **36,** 201 (1952).

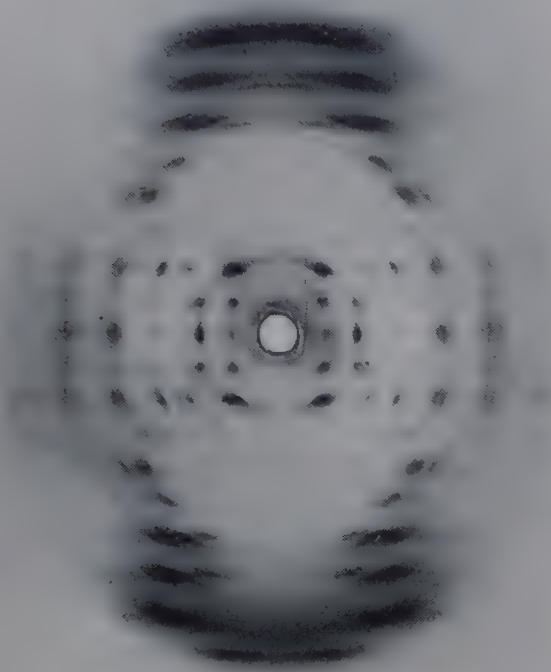

FIG. 9. X-ray diagram of the crystalline form of sodium deoxypentose nucleate (Franklin and Gosling[56]).

except when the nucleic acid had been treated in aqueous solution with acid or alkali. The titration results further show[53] that the amino group of guanine does not take part in the hydrogen-bonding in agreement with the arrangement shown in Fig. 8b.

Confirmation of the general features of the Watson and Crick formulation, which was apparently derived mainly from geometrical considerations, and more precise details concerning the dimensions of the helix and of the unit cell have been given by the X-ray data of Wilkins *et al.*[54] and Franklin and Gosling.[55-58] Fibers of the sodium salt of deoxypentose nucleic acid may be prepared[57] with a high degree of orientation by withdrawing a needle point slowly from a stiff gel of the nucleate. By suitably varying the speed of withdrawal and the water content of the gel, fibers of any diameter between 1 and 100 μ may be obtained. Such fibers give two distinct types

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[53] D. J. Cosgrove, R. H. Garner, D. O. Jordan, and S. M. Matty, in press.

[54] M. F. H. Wilkins, A. R. Stokes, and H. R. Wilson, *Nature* **171,** 738 (1953).

[55] R. E. Franklin and R. G. Gosling, *Nature* **171,** 740 (1953).

[56] R. E. Franklin and R. G. Gosling, *Nature* **172,** 156 (1953).

[57] R. E. Franklin and R. G. Gosling, *Acta Cryst.*, **6,** 673 (1953).

[58] R. E. Franklin and R. G. Gosling, *Acta Cryst.*, **6,** 678 (1953).

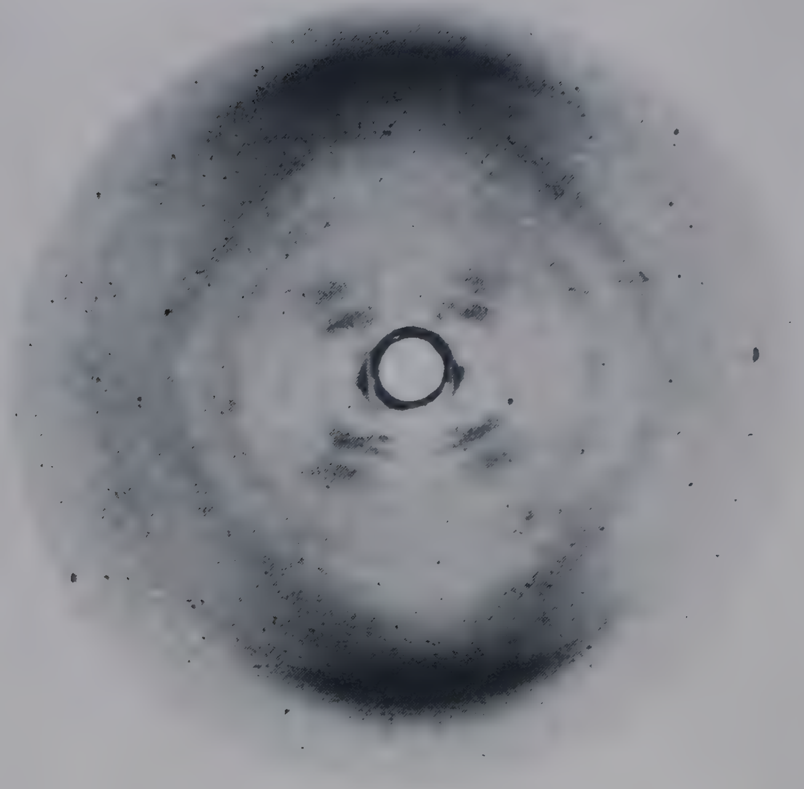

FIG. 10. X-ray diagram of the paracrystalline form of sodium deoxypentose nucleate (Franklin and Gosling[56]).

of X-ray diagram. The first (Fig. 9), which corresponds to a crystalline form (termed structure A by Franklin and Gosling[56]), is obtained in an atmosphere of about 75% relative humidity. This corresponds to a water content of the sodium nucleate of 40–45% of the dry weight. At higher humidities a different structure (B) showing a paracrystalline form with a lower degree of order appears and persists over a wide range of humidity (Fig. 10). The change from A to B is normally reversible.[57] In view of the high water content of the form B it seems reasonable to suppose that in this form the structural units of the sodium nucleate are relatively free from the influence of neighboring molecules, each unit being shielded by a sheath of water. Analysis of the X-ray diagrams of the B form of the sodium deoxypentose nucleate from *E. coli*[54] and of the sodium deoxyribonucleate of calf thymus[55] leads to a general confirmation of the helical structure although the evidence is somewhat circumstantial. Furthermore it is shown that the phosphate groups must lie on the outside of the structural unit on a helix of diameter approximately 20 A.

One of the important features of the Watson and Crick structure is that it consists of two coaxial helical chains related by a dyad axis, the third coaxial chain being absent in the nucleic acid but presumably occupied by the protein in the nucleoprotein. Direct evidence of this two-chain helix has been obtained by Franklin and Gosling,[56,58] who for structure A, which gives an X-ray diagram with sixty-six independent reflections distributed on nine well-defined layer-lines, have calculated the cylindrically averaged Patterson function (Fig. 11). The theoretical curves of the Patterson function for a smooth two-chain helix of radius 9.0 A. and in which the two chains are separated by one-half the length of the *c* axis of the unit cell are found to pass through a large proportion of the Patterson peaks. Using the cylindrical Patterson function, Franklin and Gosling determined that the unit cell is face-centered monoclinic having a = 22.0 A., b = 39.8 A., c = 28.1 A. and β = 96.5°. In order to calculate the number of nucleotides in the unit cell, it is necessary to know both the density and the water content. These are interdependent and can only be measured on a polycrystalline mass, and some doubt must rest on the values. For a density of 1.47 g./cc. at 75% relative humidity and a water content of about 40% of the dry weight, a value of twenty-three nucleotides per lattice point results, so that the unit cell will contain eleven nucleotides on each of two chains.

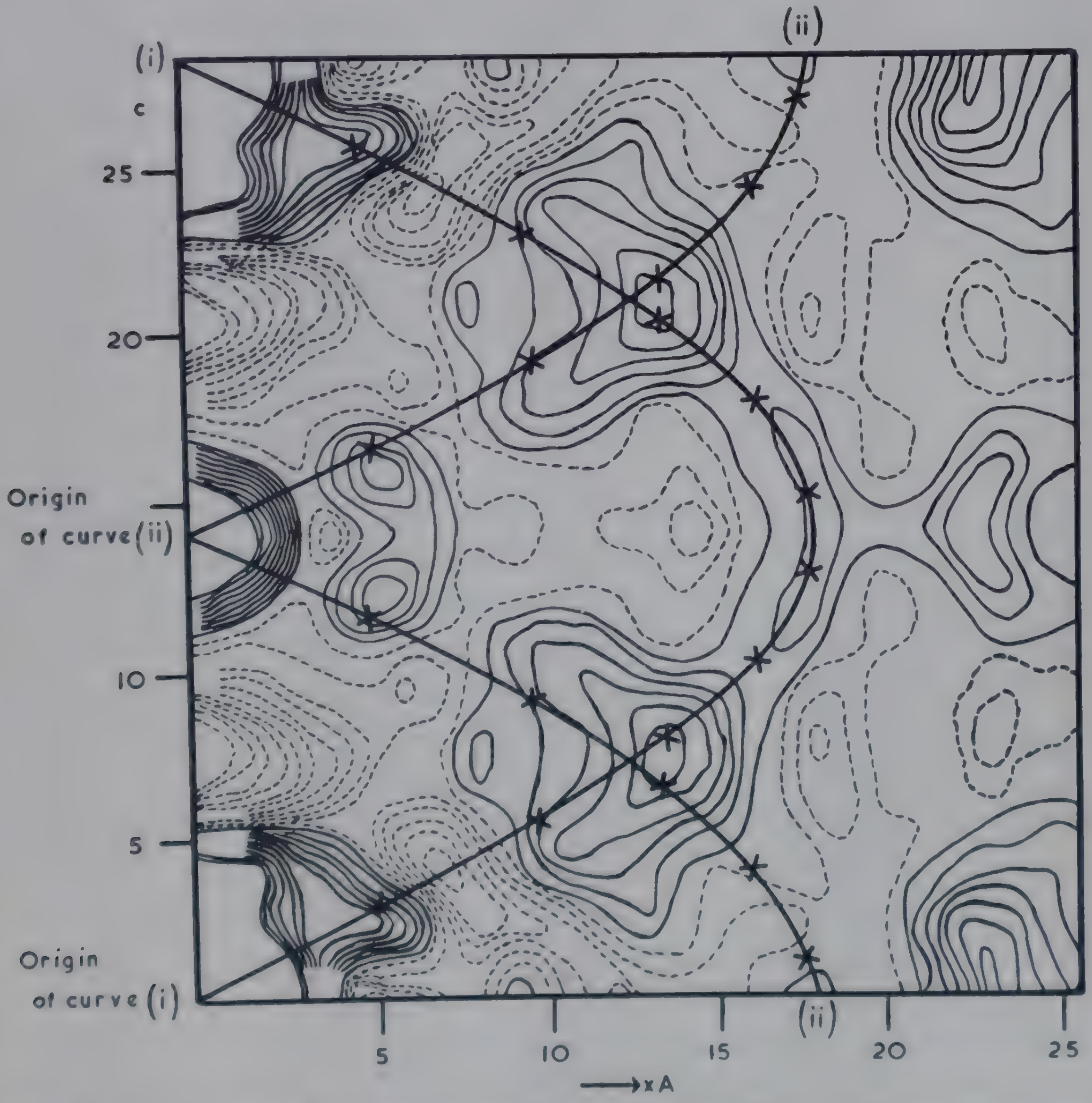

FIG. 11. Cylindrical Patterson function of crystalline sodium deoxypentose nucleate.

The complete three-dimensional Patterson function suggests that onl a part of the structure repeats itself in the plane at $c = \frac{1}{2}$ in the unit ce This is what would be expected for two coaxial chains related by a dya axis, as suggested by Watson and Crick. The phosphate groups repeat $c = \frac{1}{2}$, but since the two chains run in opposite directions, this will not true of the rest of the molecule.

These important conclusions refer only to the structure of deoxypento nucleic acids and not of ribonucleic acids. The latter acids have yet to prepared in a form that will yield satisfactory X-ray diagrams and th structural evidence based on chemical and enzymatic studies (see Cha ters 11 and 12) suggests that these acids have a branched-chain structu in marked contrast to the unbranched chain of deoxypentose nucleic acid

2. Determination of Molecular Weight

a. Deoxypentose Nucleic Acids

The apparent molecular weight (particle weight) of the deoxypento nucleate ion in solution has been measured by a variety of methods, th majority leading to a value of the order of 1×10^6. The determination the molecular weight of the nucleate ion in the ultracentrifuge is compl cated by a marked variation of the sedimentation coefficient with conce tration of the nucleate ion. This difficulty, which is typical for polyelectr lytes, may be reduced by increasing the ionic strength of the solution b the addition of neutral salt, but even so, the results shown in Fig. 12 ind cate a marked variation of the sedimentation coefficient in a buffer of ion strength 3. This dependence upon concentration necessitates the extrapol tion of the sedimentation results to obtain the value of the sedimentatio coefficient at infinite dilution. Tennent and Vilbrandt[59] observed a pa ticularly rapid increase of sedimentation constant at low concentratio which did not permit extrapolation. This observation has not been co firmed by Cecil and Ogston,[60] Atlas and Stern,[61] or by Kahler,[62] whose dat shown in Fig. 12 are in close agreement. Kahler[62] and Ogston[63] extrapolate to infinite dilution by making use of the fact that the reciprocal of th sedimentation coefficient is a linear function of the concentration of th nucleate ion at low concentrations (Fig. 13). The values obtained we 12.5×10^{-13} c.g.s. (Kahler[62]), 13.2×10^{-13} c.g.s. (Ogston[63]). These valu have been confirmed by the less detailed investigations of Conway *et al.* and Krejci *et al.*[65]

[59] H. G. Tennent and C. F. Vilbrandt, *J. Am. Chem. Soc.* **65**, 424 (1943).

[60] R. Cecil and A. G. Ogston, *J. Chem. Soc.* **1948**, 1382.

[61] S. M. Atlas and K. G. Stern, unpublished data.

[62] H. Kahler, *J. Phys. & Colloid Chem.* **52**, 676 (1948).

[63] A. G. Ogston, *Trans. Faraday Soc.* **46**, 791 (1950).

[64] B. E. Conway, L. Gilbert, and J. A. V. Butler, *J. Chem. Soc.*, **1950**, 3421.

[65] L. E. Krejci, L. Sweeny, and J. Hambleton, *J. Franklin Inst.* **248**, 177 (1949).

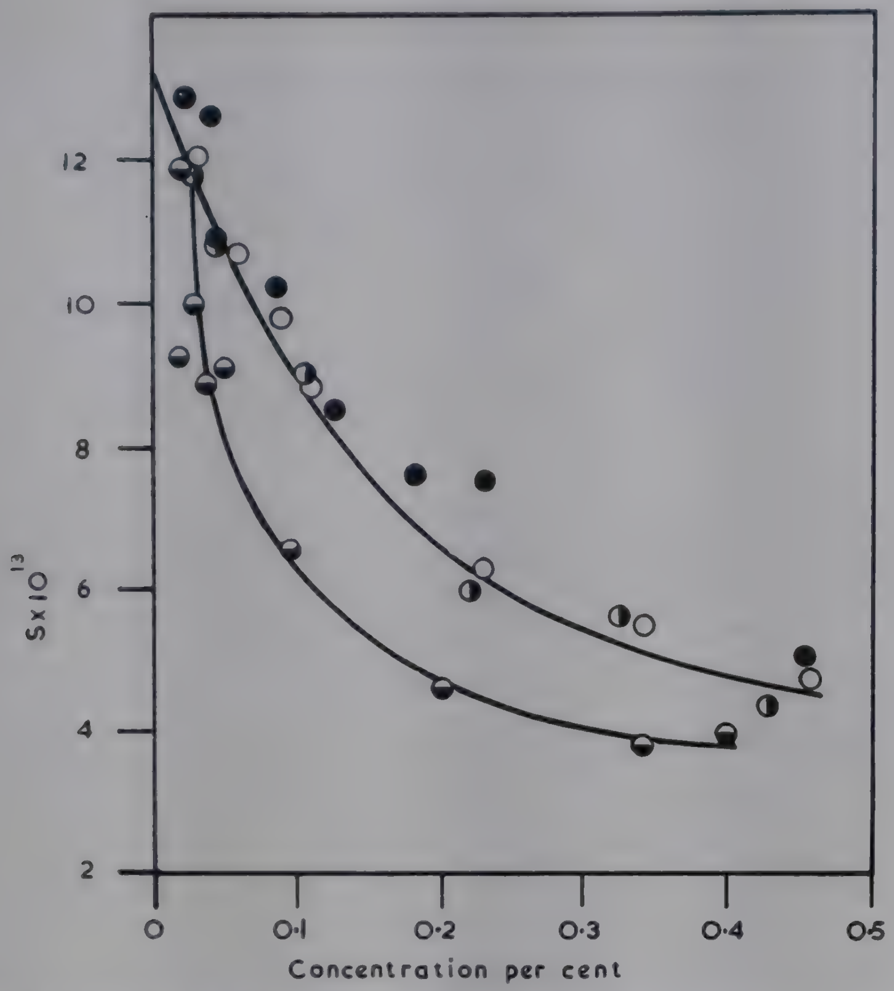

FIG. 12. The variation of sedimentation constant with concentration of sodium deoxypentose nucleate.

◑ data of Cecil and Ogston[60]
○ data of Atlas and Stern[61]
◒ data of Tennent and Vilbrandt[59]
● data of Kahler.[62]

The diffusion coefficient also varies with the concentration and although the values obtained by extrapolation to infinite dilution by Kahler[62] and Cecil and Ogston[60,63] are in fairly close agreement, the diffusion coefficient was found to vary with concentration in opposite senses by these two groups of observers. Kahler's results show a marked increase of the diffusion coefficient with concentration, while those of Cecil and Ogston show an equally marked decrease. Although the experimental technique was different in the two cases—Cecil and Ogston used the Gouy method and Kahler the more common Neurath cell with the Lamm scale or Longsworth scanning optical methods—it is clearly evident that further experimental work is desirable to determine the true concentration dependence. The determination of the diffusion coefficient is, furthermore, made more difficult, since owing to the concentration dependence of the diffusion coefficient, a skewed boundary is obtained (Fig. 14) and there may also be an apparent movement of the boundary.[66] The value of the diffusion coefficient ob-

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[66] J. A. V. Butler and D. W. F. James, *Nature* **167**, 844 (1951).

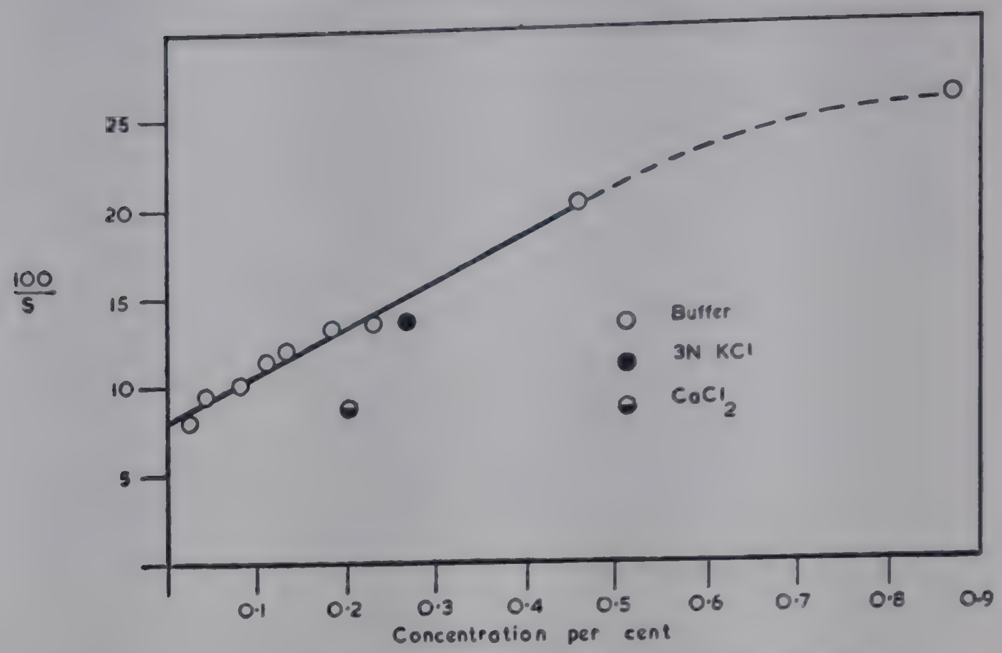

FIG. 13. Variation of $1/S$ with concentration (Kahler[62]).

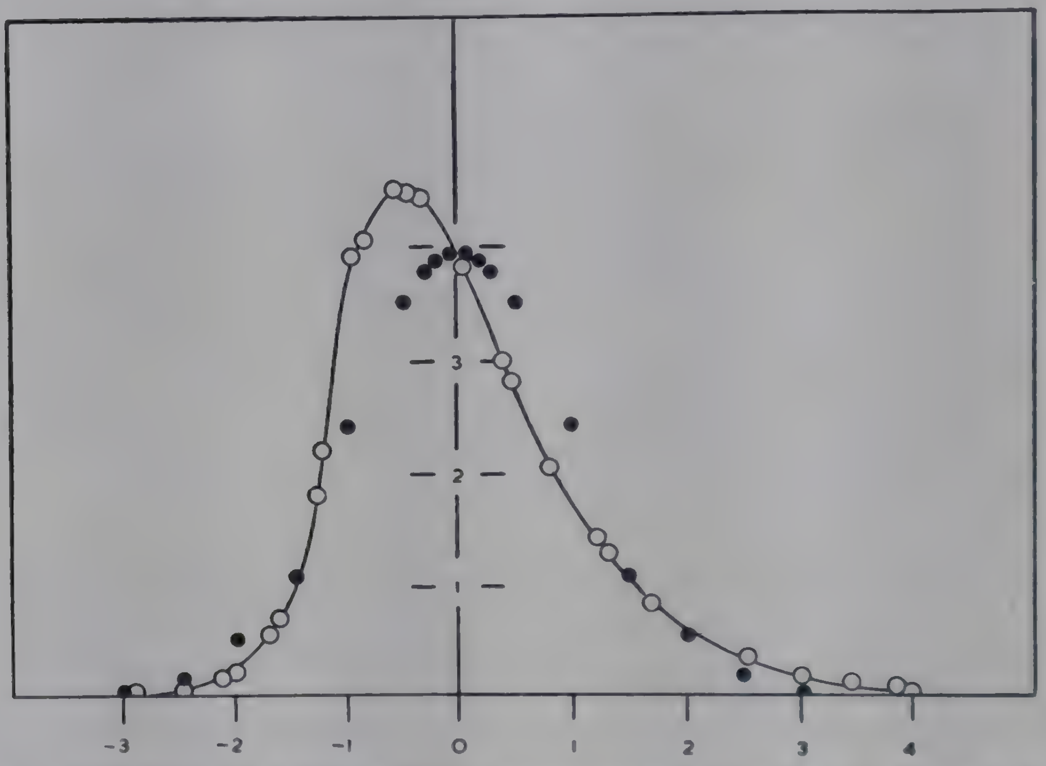

FIG. 14. Diffusion of 0.9% sodium deoxypentose nucleate in buffer at pH and 20° in normal coordinates (○). Ideal normal curve (●) (Kahler[62]).

tained by Ogston[63] was between 0.55 and 0.70 × 10^{-7} and that found Kahler,[62] 0.45 × 10^{-7}. Combination of the sedimentation and diffusion sults yield the values 1.3 × 10^{6},[60,63] 1.5 × 10^{6},[62] and 1.3 × 10^{6} [64] for di ent preparations of the sodium deoxyribonucleate of calf thymus.

In view of the difficulties attendant upon the determination of of the molecular weights of nucleic acids by sedimentation and diffu methods, other methods have been used, and probably the most accu information on the molecular weight and size and shape of the deoxypen nucleate ion in neutral solution has been obtained by Doty and Bu

TABLE IV

MOLECULAR WEIGHT AND DIMENSIONS OF DEOXYPENTOSE NUCLEATE ION IN 0.2 M NaCl

	Gulland[70]	Signer[72]	Doty
Molecular weight	4×10^6	6.7×10^6	4×10^6 7.7×10^6
Radius of gyration (A.)	1170	2200	1630
Radius for coil (A.)	2850	5400	4000
pH of solution	4.4	6.5	6.6

using the light-scattering method. Earlier studies by Oster[68] and Smith and Sheffer[69] had shown that nucleic acid solutions were suitable for such studies, and a value of the molecular weight of 4.4×10^6 was obtained by Smith and Sheffer. The values obtained by Doty and Bunce[67] for three different samples of sodium deoxypentose nucleate are given in Table IV. The sample of Gulland *et al.*[70] was identical with that examined by Cecil and Ogston[60] and shows that the light-scattering method yields a value approximately four times as great as that obtained by sedimentation. This discrepancy may be due in part to the possibility that the scattering envelope is not identical with the sedimenting unit, but is also very probably due to the value of the sedimentation coefficient obtained by existing extrapolation methods being too low. More recently, Reichmann *et al.*[71] have obtained the value of 7.7×10^6 for the molecular weight of a new sample, prepared by the method of Signer,[72] and Katz[73] has recorded a value of 8.0×10^6 for a similar preparation.

Lower estimates of the molecular weight have been obtained by Jungner *et al.*[74-76] from dielectric dispersion measurements ($1.2–8.4 \times 10^5$). For the Gulland preparation[70] the value 6.1×10^5 was obtained (compared with 1.3×10^6 by sedimentation and diffusion[60,63] and 4×10^6 by light-scattering[67] methods). The dielectric molecular weight represents the weight of individual units orienting independently in an electric field. Whether these units represent a single nucleic acid molecule or sections of a larger molecule is difficult to decide at present. Jungner *et al.* assume the former possibility to be correct and consider that the sedimentation and light-scattering methods give the molecular weight of aggregates of molecules. This point was, however, carefully investigated by Doty and Bunce,[67] who from the linearity of the zero-angle curve conclude that no aggregation occurs. It would therefore appear more probable that the dielectric molecular weight is not the true molecular weight, but that of an orienting unit.

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[67] P. Doty and B. Bunce, *J. Am. Chem. Soc.* **74,** 5029 (1952).

[68] G. Oster, *Trans. Faraday Soc.* **46,** 794 (1950).

[69] D. B. Smith and H. Sheffer, *Can. J. Research* **B28,** 96 (1950).

[70] J. M. Gulland, D. O. Jordan, and C. J. Threlfall, *J. Chem. Soc.* **1947,** 1129.

[71] M. E. Reichmann, R. Varin, and P. Doty, *J. Am. Chem. Soc.* **74,** 3203 (1952).

[72] R. Signer and H. Schwander, *Helv. Chim. Acta* **32,** 853 (1949).

[73] S. Katz, *J. Am. Chem. Soc.* **74,** 2238 (1952).

TABLE V

SOME EXAMPLES OF MOLECULAR WEIGHTS OF VARIOUS PENTOSE NUCLEIC ACIDS

Source of nucleic acid	Molecular weight	Method	Referen
Yeast	1.7×10^4	Diffusion	79
Yeast	1.03×10^4	Diffusion	80
Yeast	$1.03–2.33 \times 10^4$	Diffusion	81
Yeast	$1.7–3.5 \times 10^4$	Diffusion	82,83
Yeast	$6.0–7.0 \times 10^4$	Dielectric	84
Liver	$0.5–2.39 \times 10^4$	Diffusion and sedimentation	85
Escherichia coli	1.75×10^4		
Pancreas	1.15×10^4		
Malt	$3.45–6.97 \times 10^4$		
Tobacco mosaic virus	3.7×10^4	Diffusion and sedimentation	79
Tobacco mosaic virus	$5.9 \times 10^4–2.9 \times 10^5$		86
Rat Liver	2.64×10^5	Sedimentation	85b.

b. Pentose Nucleic Acids

The molecular weights which have been recorded for pentose nuclei acids vary from 1.31×10^3 to 2.9×10^5 according to the source, metho of preparation, and method of measurement. The low value of 1.31×1 was obtained by Myrbäck and Jorpes[77] and for many years was the mai evidence in support of the tetranucleotide hypothesis, now long discarde The experimental method employed by Myrbäck and Jorpes has bee criticized by Fletcher,[78] who considered, on a re-evaluation of their dat that the molecular weight of the sample was near 6×10^3, which althoug

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[74] G. Jungner, I. Jungner, and L-G Allgén, *Nature* **163,** 849 (1949).

[75] I. Jungner, *Acta Physiol. Scand.* **20,** *Suppl.* **69,** 1 (1950).

[76] G. Jungner, *Trans. Faraday Soc.* **46,** 792 (1950); G. Jungner and I. Jungner, *Act Chem. Scand.* **6,** 1391 (1952); G. Junger, *Acta Chem. Scand.* **6,** 1405 (1952).

[77] K. Myrbäck and E. Jorpes, *Z. physiol. Chem.* **237,** 159 (1935).

[78] W. E. Fletcher, On the Structure of Nucleic Acids, Doctoral thesis, London Univ London, England, 1948.

[79] H. S. Loring, *J. Biol. Chem.* **128,** *Sci. Proc.* **33,** 61 (1939).

[80] F. G. Fischer, I. Böttger, and H. Lehmann-Echternacht, *Z. physiol. Chem.* **271** 246 (1941).

[81] W. E. Fletcher, J. M. Gulland, D. O. Jordan, and H. E. Dibben, *J. Chem. So* **1944,** 30.

[82] I. Watanabe and K. Iso, *J. Chem. Soc. Japan* **71,** 280 (1950).

[83] I. Watanabe and K. Iso, *J. Am. Chem. Soc.* **72,** 4836 (1950).

[84] G. Jungner and L.-G. Allgén, *Acta Chem. Scand.* **4,** 1300 (1950).

[85] L. Delcambe and V. Desreux, *Bull. soc. chim. Belges* **59,** 521 (1950).

[85a] E. Volkin and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1516 (1951).

[85b] E. L. Grinnan and W. A. Mosher, *J. Biol. Chem.* **191,** 719 (1951).

[85c] E. R. M. Kay and A. L. Dounce, *J. Am. Chem. Soc.* **75,** 4041 (1953).

[86] S. S. Cohen and W. M. Stanley, *J. Biol. Chem.* **144,** 589 (1942).

still low did not correspond to the theoretical value for a tetranucleotide (1300). In view of the degradation which must generally occur during the isolation of pentose nucleic acids, it is doubtful whether the values recorded, of which examples are given in Table V, have any direct connection with the molecular weight of the parent nucleic acid in the cell and can only refer to a particular sample isolated in a certain way. Furthermore, in view of the degradation, the determinations will, in general, have been carried out on polydisperse solutions, and it is evident that an analysis for heterogeneity and a fractionation should be made prior to molecular weight determinations. Delcambe[87] and Ghuysen[88] have carried out such analyses by means of solubility measurements, and Bacher and Allen[89] have combined solubility and sedimentation studies in order to characterize pentose nucleic acids.

High-molecular-weight (greater than 10^5) nucleic acids have been obtained from virus and, more recently, from a variety of different tissues (85a, 85b, 85c). The highly polymerized ribonucleic acid obtained from tobacco mosaic virus has been studied by Cohen and Stanley.[86] The virus nucleic acid was prepared by heat denaturation of the virus and had a molecular weight of between 1.5 and 2.9×10^5. This preparation was heterogeneous, however, and decomposed spontaneously to give a nucleic acid of molecular weight $5.9\text{–}7.0 \times 10^4$, which possessed a higher degree of homogeneity. From the original highly polymeric material, by treatment with 5% sodium hydroxide, a fairly homogeneous nucleic acid sample having a molecular weight of 1.5×10^4 was obtained.

3. Acid-Base Properties

The nucleotides, as has been shown, all possess characteristic acidic dissociations owing to the presence of the primary and secondary phosphoric acid groups, the amino group, or the —NH—CO— group in the molecule. These groups again appear in the nucleic acids, and the analysis of the electrometric titration curves yields information both as to the nature of the internucleotide linkage and the macromolecular structure.

a. Deoxypentose Nucleic Acids

The early titration data on the deoxyribonucleic acid of thymus[90-97] were conflicting, the acid, in titration up to pH 8.0, being classified as pentabasic or tetrabasic by different observers and the results interpreted in terms of an open-chain and cycli structure, respectively. The samples studied, however, were considerably degrade owing to the methods of extraction that were employed, and the conflicting results hav been ascribed to the different degrees of degradation of the samples studied.[59, 98-1] The first study of an acid isolated by a mild procedure was that of Hammarsten,[1] who concluded from conductivity titrations on the free acid that the latter showe four acid dissociations for every four atoms of phosphorus, having the very approximate pK'_a values of 2.4, 3.7, 4.3, and 5.2. Incomplete electrometric titration result were obtained on similar preparations by Jorpes[102] and by Stenhagen and Teorell,[1] which indicated the absence of any appreciable secondary phosphoric acid dissociation since the solutions were found to be almost entirely unbuffered in the regio pH 6.0–9.0.

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[87] L. Delcambe, *Bull. soc. chim. Belges* **59,** 508 (1950).

[88] J. M. Ghuysen, *Bull. soc. chim. Belges* **59,** 490 (1950).

[89] J. E. Bacher and F. W. Allen, *J. Biol. Chem.* **184,** 511 (1950).

[90] H. Steudel, *Z. physiol. Chem.* **77,** 497 (1912).

[91] R. Feulgen, *Z. physiol. Chem.* **104,** 189 (1919).

[92] P. A. Levene and H. S. Simms, *J. Biol. Chem.* **65,** 519 (1925).

[93] P. A. Levene and H. S. Simms, *J. Biol. Chem.* **70,** 327 (1926).

More extensive studies on a carefully prepared high-molecular-weigh sample of the sodium salt of the deoxyribonucleic acid of calf thymus hav been made by Gulland *et al.*,[48] whose results have been confirmed by Signe and Schwander,[72] Cosgrove and Jordan,[104] and Lee and Peacocke[105] for th deoxypentose nucleic acids from lamb thymus, herring sperm, wheat germ and mouse sarcoma. The titration curve of the sodium salt of the deoxyribonucleic acid of calf thymus is shown in Fig. 15. The addition of aci or alkali to the solution in water does not at first bring about the ionizatio of groups between pH 5.0 and 11.0, but outside these limits there occurs rapid liberation of groups titrating in the ranges pH 2.0–6.0 and pH 9.0–12.0. On back-titration, either with acid from pH 12.0 or with alkali fro pH 2.5, a curve is obtained which is different from that representing th initial (forward) titration and which exhibits a well-defined point of inflection in the neutral region and shows incipient points of inflection in th regions of pH 12.0 and 2.0 corresponding, respectively, to approximatel 2.0 equivalents of alkali and 3.0 equivalents of acid for each four atoms o phosphorus. Gulland *et al.*[48] found that the same back-titration curve wa obtained irrespective of whether the titration was commenced at pH 12. or 2.5. Lee and Peacocke,[105] however, found that this was not so and tha slightly different curves were obtained on back-titrating from the differen

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[94] K. Makino, *Z. physiol. Chem.* **232,** 229 (1935).

[95] H. Bredereck, M. Köthnig, and G. Lehmann, *Ber.* **71,** 613 (1938).

[96] H. Bredereck, M. Köthnig, and G. Lehmann, *Ber.*, **72,** 121 (1939).

[97] L. Ahlström, H. von Euler, I. Fischer, L. Hahn, and B. Högberg, *Arkiv. Kemi Mineral. Geol.* **A20,** No. 15 (1945).

[98] G. Schmidt, E. G. Pickels, and P. A. Levene, *J. Biol. Chem.* **127,** 251 (1939).

[99] S. S. Cohen, *J. Biol. Chem.* **146,** 471 (1942).

[100] J. M. Gulland, G. R. Barker, and D. O. Jordan, *Ann. Rev. Biochem.* **17,** 175 (1945)

[101] E. Hammarsten, *Biochem. Z.* **144,** 383 (1924).

[102] E. Jorpes, *Biochem. J.* **28,** 2102 (1934).

[103] E. Stenhagen and T. Teorell, *Trans. Faraday Soc.* **35,** 743 (1939).

[104] D. J. Cosgrove and D. O. Jordan, *J. Chem. Soc.* **1949,** 1413.

[105] W. A. Lee and A. R. Peacocke, *J. Chem. Soc.* **1951,** 3361.

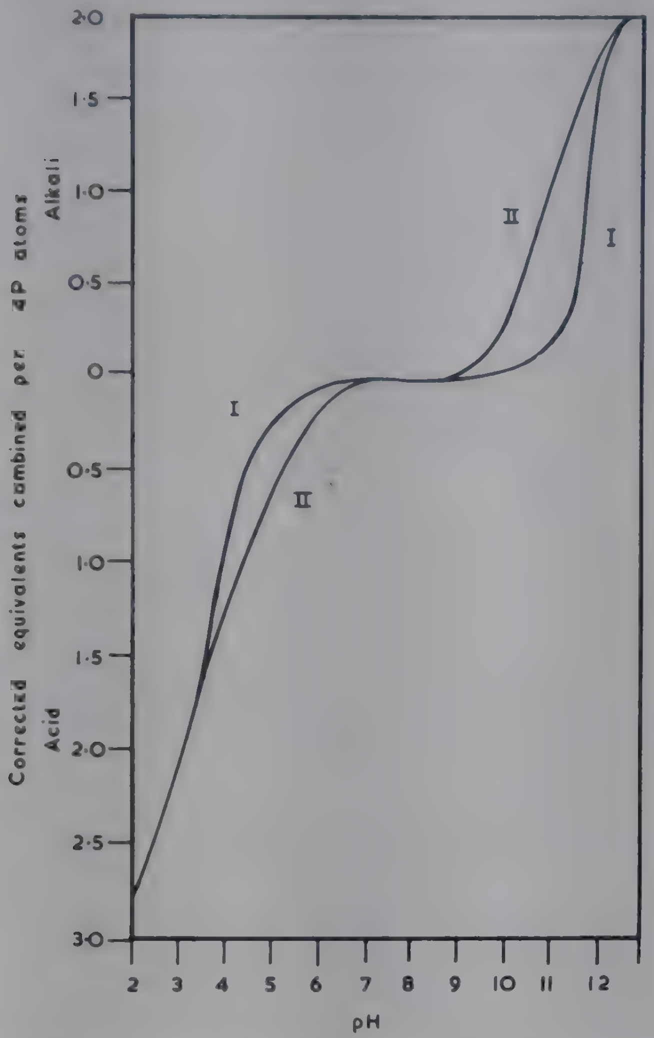

FIG. 15. The titration curve of sodium deoxypentose nucleate (Gulland, Jordan, and Taylor[48]).

I Forward titration curve
II Back-titration curve

extremes of pH (Fig. 16). Whether this discrepancy is due to a difference in drying procedure is not clear, but recent work by Garner *et al.*[106] using undried nucleic acid has confirmed the results of Lee and Peacocke.

As the acid-base characteristics of the deoxypentose nucleotides have not yet been studied, interpretation of the back-titration curve has to be made with reference to the pK'_a values of the ribonucleotides. This procedure is to some extent justified by the similarity of the acid-base properties of 9-methylxanthine and xanthosine, which suggests that the acid-base properties of the pyrimidines and purines are not dependent on the nature of the substituent radical in the 9(or 3)-position so long as it is a nonresonating system and that the glycosidic, C—N, link remains a single bond. The titration curve (Figs. 15 and 16) shows that two main types of titratable group exist in the molecule: that titrating in the range pH 2.0–6.0 and that titrating in the alkaline range pH 8.0–12.0. From the discussion of the dissociating groups in the nucleotides it is evident that these groups are the

[106] R. H. Garner, D. O. Jordan, and S. M. Matty, unpublished results.

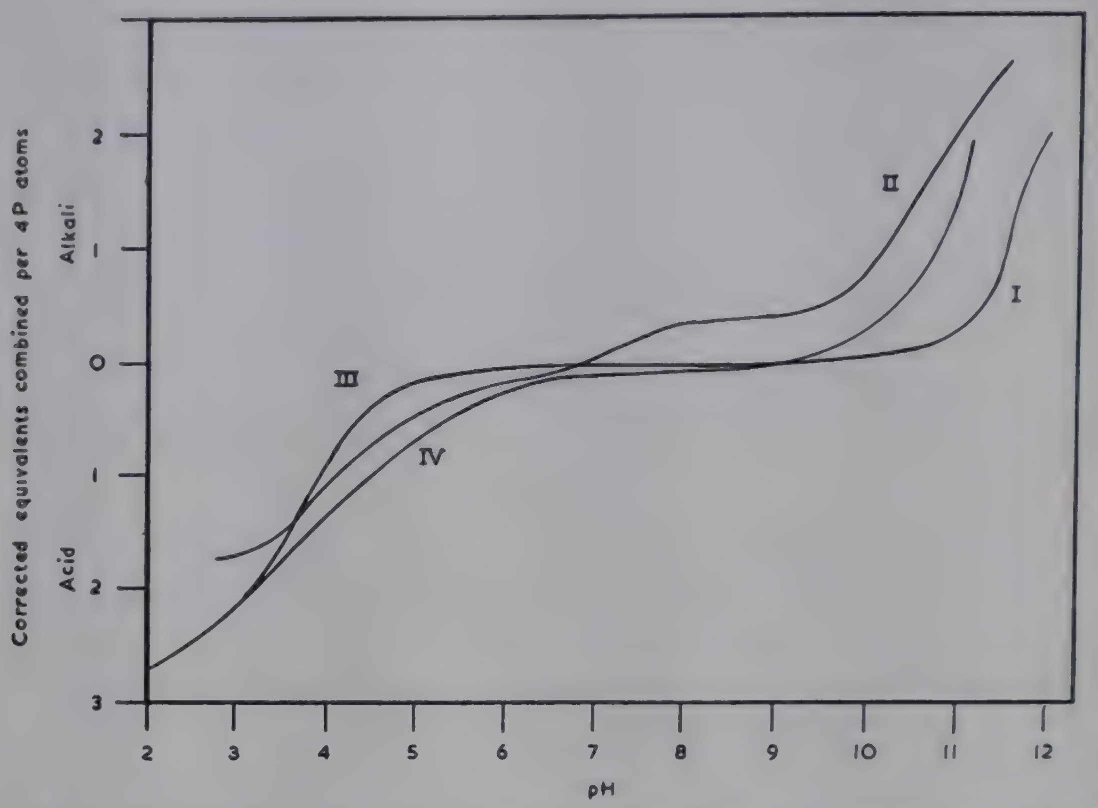

FIG. 16. The titration curve of sodium deoxypentose nucleate (Lee and Peacocke[105]).

I Titration with alkali from pH 6.4 to 12.0
II Back-titration with acid from pH 12.0
III Titration with acid from pH 6.4 to 2.4
IV Back-titration with alkali from pH 2.4

purine–pyrimidine amino and the —NH—CO— dissociations of guanine and thymine, respectively. The number of these dissociating groups is known with some accuracy from the analytical results of Chargaff[50] and Wyatt[51,52] [cf. Chargaff, Chapter 10], and in Table VI the results of Lee and Peacocke[105] are summarized for three deoxypentose nucleic acids. The analysis[48] of the sodium salt of the deoxyribonucleic acid from calf thymus for sodium shows that there is one sodium ion for every phosphorus atom, and, in view of the fact that the amount of the secondary phosphoric acid dissociation is small (Table VI), these must be combined largely or entirely with the primary phosphoric acid dissociations. The presence of the theoretical number of amino, primary phosphoric acid, and —NH—CO— dissociations is in agreement with the view that the deoxypentose nucleic acids have a long-chain structure in which the internucleotide bond is through a phosphoester linkage. The difficulty in accepting this type of linkage in view of the very different behavior of deoxypentose nucleic acids, compared with ribonucleic acids, towards alkali has now been overcome by the conclusion of Brown and Todd[107] that the lability of the latter acid is due to the ease of formation of a cyclic intermediate involving the adjacent

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[107] D. M. Brown and A. R. Todd, *J. Chem. Soc.* **1952,** 52.

TABLE VI

THE TITRATABLE GROUPS OF THE SODIUM SALTS OF SOME DESOXYPENTOSE NUCLEIC ACIDS

Groups		Calf thymus	Herring sperm	Wheat germ
1. Amino (guanine)	pK'_a	2.5	2.35	2.9
	Equivalents/4P	0.75	0.71	0.66
2. Amino (adenine)	pK'_a	3.7	3.7	3.85
	Equivalents/4P	1.02	1.0	0.9
3. Amino (cytosine and 5-methylcytosine[a])	pK'_a	4.75	4.85	4.5
	Equivalents/4P	0.84	0.92	0.84
4. Secondary phosphoric acid dissociation	pK'_a	6.5	6.5	5.5
	Equivalents/4P	0.33	0.18	0.42
5. —NH—CO— dissociation of guanine and thymine	pK'_a	10.4,11.4	10.4,11.4	—
	Equivalents/4P	1.76	1.76	1.57

[a] pK'_a of 5-methylcytosine assumed the same as that of cytosine.

sugar hydroxyl group to that bearing the phosphate group [cf. *Brown and Todd*, Chapter 12.]. The presence of a small amount of secondary phosphoric acid dissociation can only indicate that there is some chain-branching, which will occur every ten or twenty nucleotides. This view is confirmed by the dye-adsorption measurements of Cavalieri and Angelos,[108] who, in order to explain the experimental results, found it necessary to assume the existence of two binding sites. The ratio of these sites is lower than that found from titration, being one in thirty.

The initial (forward) titration curve of the high polymeric deoxypentose nucleic acids is anomalous in that on the addition of acid or alkali to the solution in water, no groups are titrated at first between pH 5.0 and 11.0, but outside these limits there occurs a rapid ionization of the amino groups and the —NH—CO— dissociations. This general behavior was first observed by Gulland *et al.*[48] for the acid from calf thymus and has been confirmed by electrometric titration of other acids and for different methods of preparation by Signer and Schwander,[72] Cosgrove and Jordan,[104] and Lee and Peacocke.[105] It has also been confirmed by spectrophotometric titration by Shack and Thompsett.[109] The ionization of groups at pH 11.5 and in the range pH 3.5–4.5 is accompanied by a marked fall in the viscosity[110] and the disappearance of streaming birefringence.[101,110,111] This decrease in th viscosity on the addition of acid and alkali was considered by Vilbranc and Tennent[111] to be caused by depolymerization, which slowly reverse when the solution was returned to neutrality. Gulland *et al.*,[48] howeve have shown that this depolymerization cannot involve the rupture of th internucleotide phosphoester linkages since there is no increase in th amount of secondary phosphoric acid dissociation on back-titration, an these authors suggested that some of the amino groups of the pyrimidine and purines are linked by hydrogen bonds to the —NH—CO— dissociations; the ionization of either group would then bring about the liberatio of both as shown by the position of the back-titration curve (Figs. 15 an 16). The liberation of the groups at pH values more alkaline or acid tha would be expected from the normal pK'_a values of the —NH—CO— an amino groups, respectively, will be due, most probably, to the stability o the nucleic acid hydrogen-bonded structure and the need to break severa of the hydrogen bonds simultaneously. The ionization would thus b shifted in the direction of higher hydrogen and hydroxyl ion concentration as is shown by the titration curve.

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[108] L. F. Cavalieri and A. Angelos, *J. Am. Chem. Soc.* **72,** 4686 (1950).

[109] J. Shack and J. M. Thompsett, *J. Biol. Chem.* **197,** 17 (1952).

[110] J. M. Creeth, J. M. Gulland, and D. O. Jordan, *J. Chem. Soc.* **1947,** 1141.

From the titration data alone it is not possible to decide whether th bonds are intra- or intermolecular. The marked decrease in viscosity whicl has been observed by Creeth *et al*,[110] at almost the same pH values as th ionization of the groups would serve to indicate that the bonds join smalle molecular units, which in solution show lower viscosity than the hydrogen-bonded macromolecules. The sedimentation data,[60] although not conclusiv on this point, tend to confirm this conclusion.

These conclusions, drawn from titration results, are in remarkable agreement with the structure proposed by Watson and Crick.[49] The hydroger bonds between the single nucleic acid chains involve the amino groups o adenine and cytosine and the —NH—CO— groups of guanine and thymine Furthermore, more careful examination of the titration curve[53] shows tha the anomalous behavior ceases at approximately pH 3.5 after the titratio of 1.8–2.0 equivalents of amino group, i.e., the back- and forward-titratio curves are coincident at pH values more acid than 3.5, which indicates tha the most acid amino group, viz., that of guanine, does not partake in the formation of hydrogen bonds as suggested by Watson and Crick.

b. Pentose Nucleic Acids

The titration of pentose nucleic acids has yielded important informatio concerning the nature of the ionizable groups and of the internucleotid linkage. No information concerning the macromolecular structure has bee obtained, however, since the samples studied have all been isolated by us of fairly strong reagents and the nucleic acid has consequently suffered degradation. It is not known, therefore, whether the pentose nucleic acids exist in a similar hydrogen-bonded structure to that of the deoxypentose nucleic acids.

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[111] C. F. Vilbrandt and H. G. Tennent, *J. Am. Chem. Soc.* **63**, 1806 (1943).

Early work by Levene and Simms,[93] Jorpes,[102] Makino,[94] and Bredereck *et al.*[112,113] had shown that the ribonucleic acid of yeast contained three to four ionizable groups per four atoms of phosphorus on titration to pH 8.0. The first systematic study was that of Allen and Eiler,[114] who were the first to observe that the titration curve indicated the presence of three amino groups for every four atoms of phosphorus, together with one group titrating in the range pH 5.0–7.5 and which they regarded as a primary phosphoric acid group that had been weakened by virtue of the ionization of the remaining phosphoric acid dissociations. Fletcher *et al.*,[115] who obtained titration curves (Fig. 17) very similar to those of Allen and Eiler, interpreted their results as indicating the presence, in the ribonucleic acid of yeast, of three amino groups, one secondary phosphoric acid, two —NH—CO—, and three primary phosphoric acid dissociations for every four atoms of phosphorus. The pK'_a values of the nucleotides were assumed to be unchanged in the nucleic acid. This conclusion was confirmed by the titration curve of the deaminated acid (Fig. 17); the removal of the amino groups permits the direct titration of the primary phosphoric acid dissociations, and the curve indicates the presence of three primary and one secondary phosphoric acid dissociations, the —NH—CO— dissociation of xanthosine which titrates in the range pH 5.0–7.0 (pK'_a 6.0), and three —NH—CO— dissociations titrating in the range pH 8.0–12.0. These results suggest that, on average, three secondary and one primary phosphoric acid groups are utilized in yeast ribonucleic acid for every four atoms of phosphorus, in forming the phosphoester internucleotide bond. This conclusion indicates that one in every four atoms of phosphorus (on average) is triply esterified. The shape of the electrometric titration curve has been confirmed by several workers,[116-122], who have generally interpreted their results in the same way although there is no general agreement about the

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[112] H. Bredereck and M. Köthnig, *Ber.* **72,** 121 (1939).

[113] H. Bredereck and I. Jochman, *Ber.* **75,** 395 (1942).

[114] F. W. Allen and J. J. Eiler, *J. Biol. Chem.* **137,** 757 (1941).

[115] W. E. Fletcher, J. M. Gulland, and D. O. Jordan, *J. Chem. Soc.* **1944,** 34.

[116] H. Chantrenne, *Bull. soc. chim. Belges* **55,** 5 (1946).

[117] H. Chantrenne, K. Linderstrøm-Lang, and L. Vandendriessche, *Nature* **159,** 877 (1947).

[118] L. Vandendriessche, *Compt. rend. trav. lab. Carlsberg, Ser. chim.* **27,** 341 (1951).

[119] Y. Khouvine and J. Gregoire, *Bull. soc. chim. biol.* **26,** 424 (1944).

[120] C. A. Zittle, *J. Biol. Chem.* **166,** 491 (1946).

[121] G. Wiener, E. L. Duggan, and F. W. Allen, *J. Biol. Chem.* **185,** 163 (1950).

[122] L. F. Cavalieri, S. E. Kerr, and A. Angelos, *J. Am. Chem. Soc.* **73,** 2567 (1951).

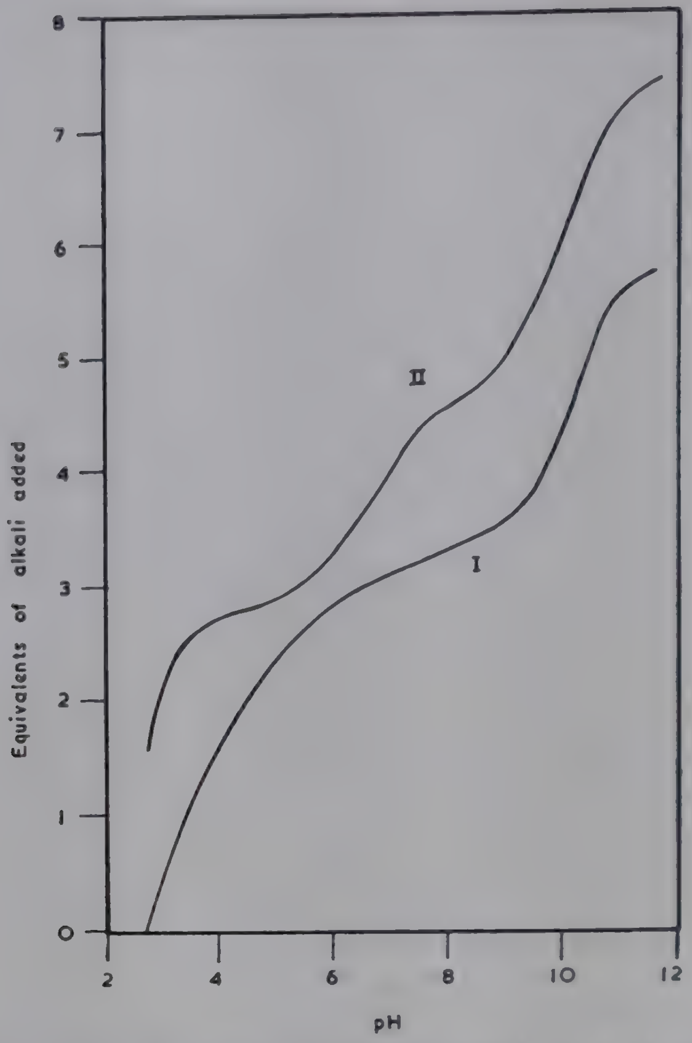

FIG. 17. Titration curve of yeast ribonucleic acid, I, and of deaminated yeas ribonucleic acid, II (Fletcher, Gulland, and Jordan[115]).

relative amount of the secondary phosphoric acid dissociation and thi may well vary both with the method of preparation and the source of th nucleic acid. The presence of a triply esterified phosphorus in the poly nucleotide necessarily involves the formation of a branched-chain structure It is not possible at present to say to which nucleotide the triply esterifie atom belongs; Cavalieri *et al.*,[122] in ascribing it to uridylic acid, have mad the same mistake as Fletcher *et al.*,[115] later corrected by Gulland *et al.*,[48] i that they have regarded the nucleotides containing amino groups as bein in the nonzwitterionic form.

The conclusions derived from electrometric titration data have been confirmed b the dye-adsorption studies of Cavalieri *et al.*[122, 123] In their studies of the adsorptio of rosaniline by pentose nucleic acid using the method of equilibrium dialysis, the found that their results did not follow the simple equation: $r/c = kn - kr$ (where is the amount of dye bound per mole of nucleic acid, c is the equilibrium concentra tion of the dye, $n = r_{\max.}$, and k is the binding constant), but it was found necessary t assume two types of binding sites in the nucleic acid and the equation $r/c = n_1k_1/(1 + k_1c) + n_2k_2/(1 + k_2c)$ was found to describe the experimental results. Since rosanilin

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[123] L. F. Cavalieri, A. Angelos, and M. E. Balis, *J. Am. Chem. Soc.* **73**, 4902 (1951).

is a cationic dye, it will be adsorbed only at negatively charged sites, and at neutral pH these will be the phosphoric acid dissociations.

A further possibility of testing these conclusions lies in the different stability of the tertiary and secondary esters of phosphoric acid towards alkaline reagents.[124] The alkaline hydrolysis constants have been given by Cavalieri[125] as being *ca.* 3.5×10^{-2} min.$^{-1}$ for the reaction: triester phosphate → diester phosphate, and *ca.* 2.9×10^{-2} min.$^{-1}$ for the reaction: diester phosphate → monoester phosphate. Cavalieri's results of a kinetic study[125] of the alkaline hydrolysis of yeast ribonucleic acid again suggests that there are at least two types of phosphate linkage, one more labile than the other. The analysis of the kinetic data is, however, difficult in view of possible differences in the rate of hydrolysis of bonds between different nucleotides, and Cavalieri did not consider it justifiable to relate the two types of linkage definitely to the tri- and diester phosphates.

4. THE SOLUTION PROPERTIES OF SODIUM DEOXYPENTOSE NUCLEATE

Although studies of the properties and structure of nucleic acids and their sodium salts in the solid state have yielded results of fundamental importance, it is, nevertheless, the size, shape, and rigidity of the nucleate ion in solution which is probably of greatest interest from the biological viewpoint. In view of the very different nature of the environments, conclusions drawn from studies of the solid state may require some modification when the properties in solution are considered. It has been known for some time that the properties of the nucleate ion in solution are very dependent on the pH and ionic strength of the solvent as well as upon the previous treatment of the nucleic acid, and the nature of these changes will now be considered.

Owing to the difficulty in obtaining high-molecular-weight preparations of pentose nucleic acids, most studies have been made on the sodium salt of a deoxypentose nucleic acid and this discussion will be confined to the properties of this group of acids.

a. The Influence of Ionic Strength in Neutral Solution

Measurements of viscosity and streaming birefringence made on solutions of sodium deoxypentose nucleate containing no added electrolyte have not been found to be reproducible (see for example Sadron[126]), particularly at concentrations above about 0.005%, the actual value varying with the nucleate sample, being lower for high-molecular-weight preparations. This lack of reproducibility will be due to the slow degradation of the nucleate which occurs in aqueous solution and which is accelerated by increase of temperature and retarded by the presence of electrolyte.[127] It could also be caused by aggregation of the ions to form ionic micelles in the more concentrated solutions. A further cause of the nonreproducibility is that the properties vary considerably with the method of preparation of the solution, and the most satisfactory method is to permit the fibrous nucleic acid to dissolve slowly without stirring at *ca.* 0°, when solution is normally complete within twelve hours. Solutions of sodium deoxypentose nucleate in pure water as solvent and prepared in this way show a very high viscosity, which is dependent to a marked degree on the rate of shear, and also exhibit strong streaming birefringence. On the addition of electrolyte all three effects are considerably reduced and the reproducibility of the measurements greatly increased.

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[124] G. M. Kosolapoff, "Organophosphorus Compounds," p. 232. John Wiley and Sons, Inc. New York, 1950.

[125] L. F. Cavalieri, *J. Am. Chem. Soc.* **73,** 4899 (1951).

[126] C. Sadron, *Prog. Biophys. and Biophys. Chem.* **3,** 237 (1953).

The decrease of viscosity on the addition of electrolyte[72,110,128,129] was first explained by Greenstein and Jenrette[128] as a reversible depolymerization of the deoxypentose nucleate. However, comparison of the acid-base properties in 1.0 *M* potassium chloride solution with those in pure water as solvent[48] shows that there is no increase in the titratable acidic and basic groups when the deoxypentose nucleate is in the former solvent and furthermore indicates that the hydrogen-bonded structure is not affected by the increase in ionic strength. More definite evidence that the process is not one of depolymerization is afforded by the light-scattering measurements of Reichmann *et al.*[71] which show that an increase of ionic strength does not change the molecular weight, but only produces relatively small changes in the shape of the ion. The similarity of the behavior of the deoxypentose nucleate ion and of synthetic polyelectrolytes such as poly-*N*-*n*-butyl-4-vinylpyridonium bromide to changes of ionic strength, led Jordan[130] to suggest that in water solution the nucleate ion was fully stretched by virtue of the repulsion between the charged ($\rightarrow PO^-$) groups. The fall in viscosity on the addition of electrolyte would then be produced by a coiling of the molecule permitted by a neutralization of these charged groups. This mechanism is identical with that suggested by Fuoss and Strauss[131] for the synthetic polyelectrolytes. The early work of Bungenberg de Jong and Kwan[132] had indicated that there was a similarity between the viscosimetric behavior of nucleic acids and polyelectrolytes, and this has been confirmed by Basu.[133] In a more recent study, however, Pouyet[134] has made measurements of the

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[127] T. Miyaji and V. E. Price, *Proc. Soc. Exptl. Biol. Med.* **75,** 311 (1950).

[128] J. P. Greenstein and W. V. Jenrette, *J. Natl. Cancer Inst.* **1,** 77 (1940).

[129] G. Vallet and H. Schwander, *Helv. Chim. Acta* **32,** 2508 (1949).

[130] D. O. Jordan, *Trans. Faraday Soc.* **46,** 792 (1950).

[131] R. M. Fuoss and U. P. Strauss, *J. Polymer Sci.* **3,** 246; 602 (1948).

[132] H. G. Bungenberg de Jong and U. S. Kwan, *Kolloidchem. Beih.* **31,** 89 (1930).

[133] S. Basu, *Nature* **168,** 341 (1951).

[134] J. Pouyet, *Compt. rend.* **234,** 152 (1952).

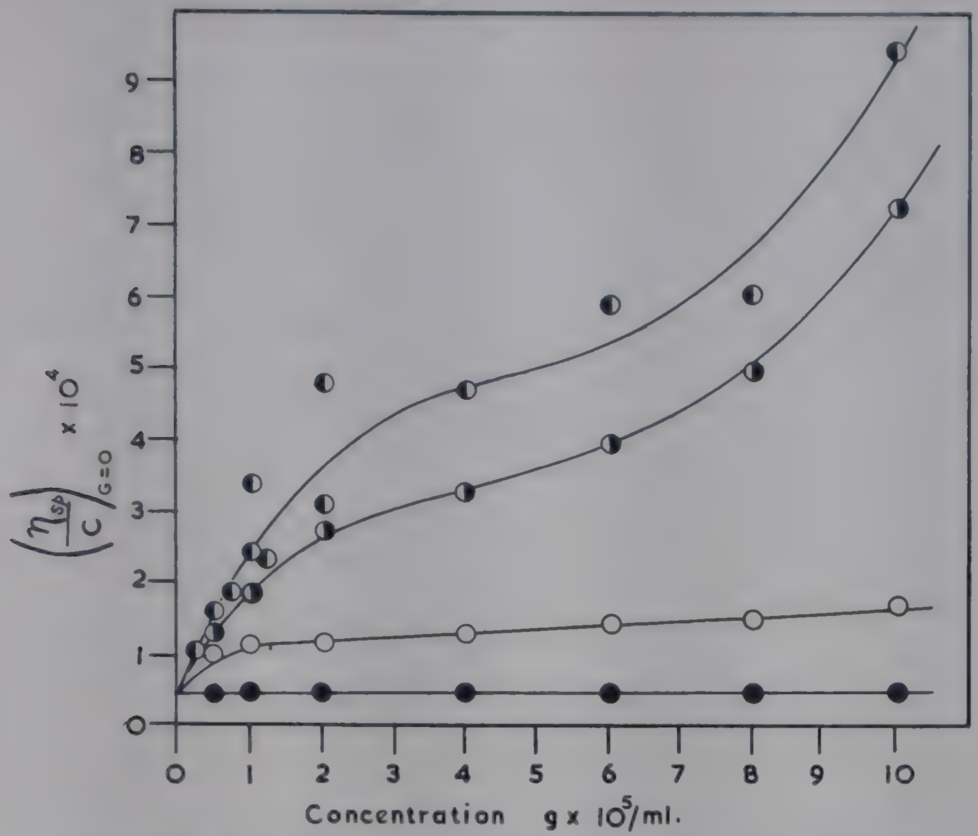

FIG. 18. Variation of $\eta_{sp.}/c$ with concentration of sodium deoxypentose nucleate at different concentrations of sodium chloride (Conway and Butler[135]).

● 10^{-1} *N* ○ 10^{-3} *N*
◑ 10^{-4} *N* ◐ No added salt

viscosity of deoxypentose nucleate solutions at low rates of shear in a Couette viscometer and has interpreted his results as indicating that at infinite dilution of nucleate there is no influence of ionic strength on the viscosity of these solutions. This view has been supported by the more extensive results of Conway and Butler,[135] who determined viscosities at sufficiently low rates of shear to permit extrapolation to zero shear (Fig. 18). These conclusions would indicate that the deoxypentose nucleate ion does not show a contraction, as was suggested by Jordan,[130] on the addition of electrolyte, and Conway and Butler[135] consider that the variation of viscosity is due entirely to electrostatic interaction between the nucleate ions, which would be reduced by the addition of sodium ions.

The problem of the fall in viscosity on the increase of ionic strength is clearly intimately related to the rigidity of the deoxypentose nucleate ion. Further evidence on this problem comes from streaming birefringence studies.[136-141] Schwander and Cerf[137] find that the streaming birefringence

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[135] B. E. Conway and J. A. V. Butler, *J. Polymer Sci.*, **12,** 199 (1954).

[136] O. Snellman and G. Widström, *Arkiv Kemi Mineral. Geol.* **A19,** No. 31 (1945).

[137] H. Schwander and R. Cerf, *Helv. Chim. Acta* **32,** 2356 (1949).

[138] H. Schwander, *J. chim. phys.* **47,** 718 (1950).

[139] H. Schwander and R. Cerf, *Helv. Chim. Acta* **34,** 436 (1951).

[140] H. Schwander and R. Cerf, *Experientia* **7,** 95 (1951).

[141] H. Schwander and R. Signer, *Helv. Chim. Acta* **34,** 1344 (1951).

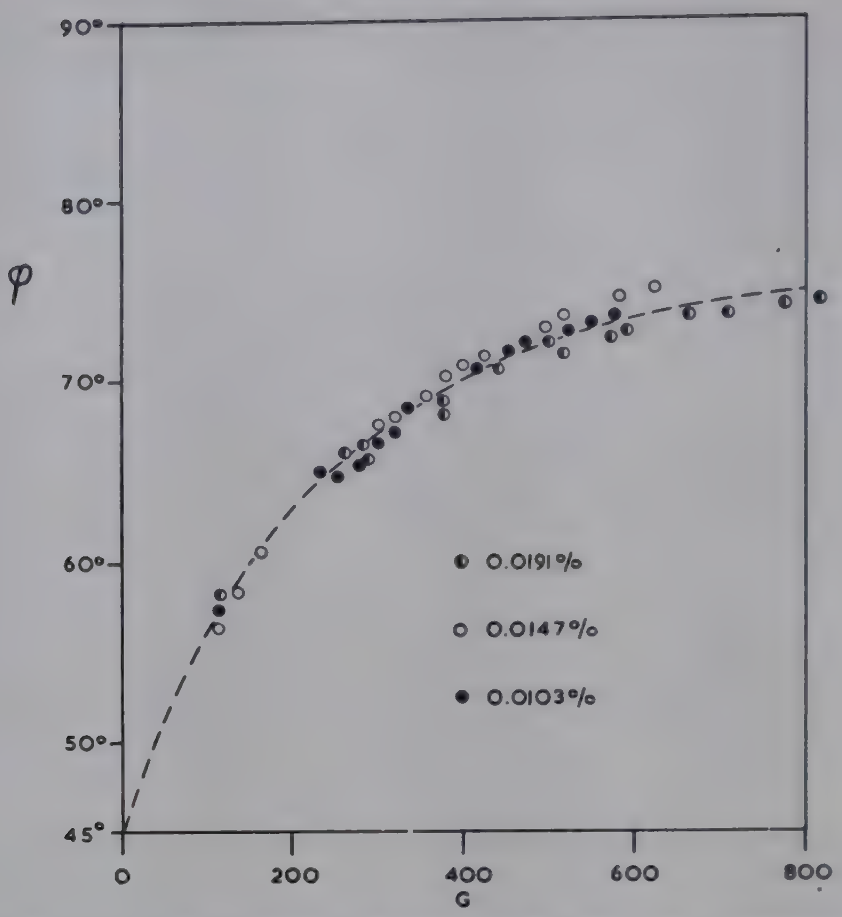

FIG. 19. Streaming birefringence of solutions of sodium deoxypentose nucleate in 10% sodium chloride (Schwander and Cerf[137]).

decreases with ionic strength, but the rotational diffusion constant is apparently independent of both ionic strength and nucleate concentration (Fig. 19) and has a value of 36.6 sec.$^{-1}$. From these results and the magnitude of the birefringence, Sadron[126] has concluded that the particles are very long and not capable of contraction. The experiments of Schwander and Cerf[139] in which the birefringence was measured using solutions of sodium deoxypentose nucleate in which the viscosity was varied by adding glycerol, are, however, probably of greater significance. The birefringence may arise either from the orientation of a rigid particle in the stream lines or from the deformation of a random coil to give increased asymmetry followed by orientation; the contribution of these two effects may be analyzed by varying the viscosity of the solvent (see Cerf[142] for theoretical details). On increasing the viscosity of the solvent, tan α (where α is the initial slope of the curve relating extinction angle and velocity gradient) should be a linear function of the solvent viscosity if the particles are rigid. Schwander and Cerf[139] and Sadron[126] first interpreted these results (Fig. 20) as indicating that the nucleate ion was a rigid particle, the discrepancy at higher values of the viscosity being attributed to heating effects. On the basis of a more detailed theoretical treatment of the problem, Cerf[142] considers that the change of slope at higher viscosities (Fig. 20) is due to a de-

[142] R. Cerf, *J. Polymer Sci.*, **12**, 15 (1954).

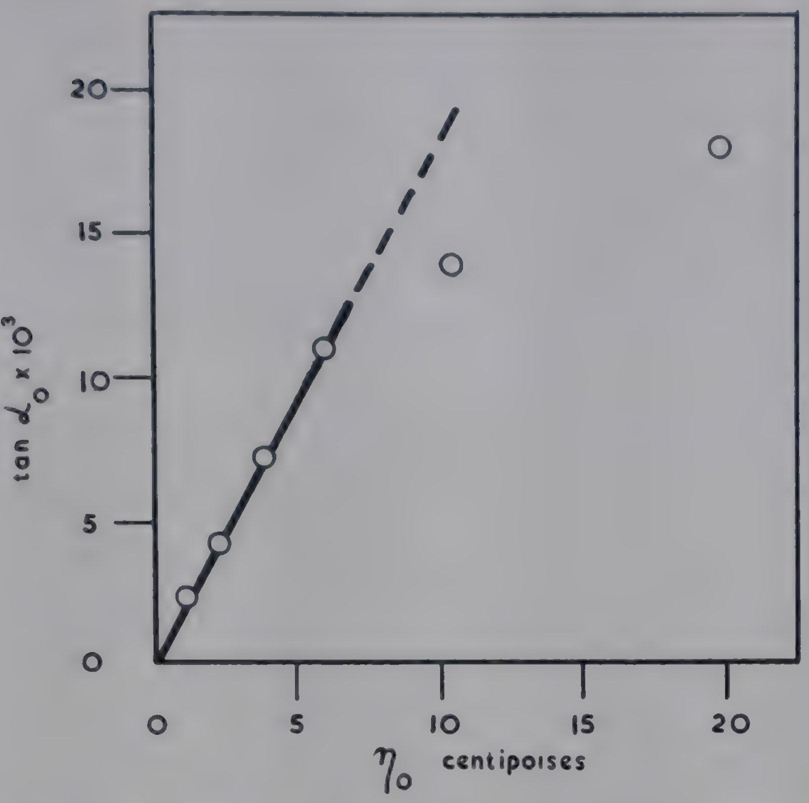

FIG. 20. Values of tan α_0 for a solution of sodium deoxypentose nucleate with sodium chloride when viscosity is varied by addition of glycerine (Schwander and Cerf[139]).

formation of the nucleate ion, and the more detailed experimental study of Horn *et al.*[143] confirms this view although the neutral ion in neutral solution is much less deformable than the acid-treated material.

The light-scattering results of Reichmann *et al.*[71] and of Rowen *et al.*[144] confirm the conclusion that the deoxypentose nucleate ion is deformable, but the contraction on the addition of electrolyte to a water solution is much less than for a typical polyelectrolyte. Thus Rowen *et al.*[144] found that the maximum dimension decreased from 6800 A. to 4500 A. when the ionic strength was increased from 0 to 2×10^{-2}. A similar change of solvent with sodium polymethacrylate would produce about a tenfold decrease in the maximum dimension.[145] Furthermore, Reichmann *et al.*[71] conclude that the deoxypentose nucleate ion in 0.2 *M* sodium chloride solution is only slightly more asymmetric than that of a random coil; if this be so, the streaming birefringence can only arise by a deformation of the molecule.

Direct evidence that sodium ions are bound to the nucleate ion has been obtained from determinations of the charge on the nucleate ion at various ionic strengths. The charge has so far only been determined from measurements of the membrane potential, and the measurements of Creeth and Jordan[146] have recently been extended by Shack *et al.*[147] Their results are very similar in that the charge on the nucleate ion at the ionic strengths studied is less than the theoretical value for the fully charged ion, thus indicating a considerable degree of ion-pair formation. Shack *et al.*[147] observe only a small variation of the charge with ionic strength, the average value in the range 0.005 and 0.1 being 1.89 negative charges for every four atoms of phosphorus. The variation of charge observed by both Creeth and Jordan and Shack *et al.* was an increase of negative charge with increase of ionic strength. The reason for this variation is not clear, and, in view of the limitations and inaccuracies of the membrane-potential method, it is clearly desirable that the charge should be investigated by other methods.

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[143] P. Horn, J. Leray, J. Pouyet, and C. Sadron, *J. Polymer Sci.* **9,** 531 (1952).

[144] J. W. Rowen, M. Eden, and H. Kahler, *Biochim. et Biophys. Acta* **10,** 89 (1953).

[145] A. Oth and P. Doty, *J. Phys. & Collid Chem.* **56,** 43 (1951).

[146] J. M. Creeth and D. O. Jordan, *J. Chem. Soc.* **1949,** 1409.

Changes in the ultraviolet absorption spectrum on the addition of electrolyte which have been studied systematically by Thomas,[148] Shack *et al.*,[149] and Lawley[150] are also consistent with the view that the nucleate ion is capable of some deformation. These authors find that the value of the extinction coefficient at 260 mμ is lowered by the addition of electrolytes, the cations having a specific effect which is dependent upon their charge. The adsorption of Na^+, K^+, Mg^{++}, Ba^{++}, and Zn^{++} probably occurs at the phosphate groups and is nonspecific since the overall shape of the adsorption band is not affected. H_3O^+ and Ag^+ ions, however, appear to have a more specific action since the shape of the absorption curve changes and $\lambda_{max.}$ shifts to longer wavelengths. The lowering of the absorption is unlikely to be due to a shadowing effect and is more probably a true difference in the absorption of the ring systems due to changes in the configuration of the rings in the macro-ion. In the Watson and Crick structure[49] the extent of stretching of the polynucleotide chain is determined by an equilibrium between the hydrogen-bonding and the Van der Waals forces on the one hand and the repulsion between the charged groups on the other. A decrease of the latter due to ion-pair formation will permit a small decrease in the length of the ion and, owing to the new relative configurations of the purine and pyrimidine rings, a change in the absorption spectrum. [Cf. *Beaven, Holiday, and Johnson*, Chapter 14.]

b. Conclusions as to the Size and Shape of the Deoxypentose Nucleate Ion in Neutral Solution

The problem of determining the dimensions of a polyelectrolyte of unknown macro-structure is a most exacting one, as the small number of successful studies with synthetic polyelectrolytes of known structure testifies. However, from the experimental evidence discussed above it is possible to draw some conclusions concerning the size and shape of the deoxypentose nucleate ion. Such conclusions must, as yet, be tentative owing to the marked disagreement which exists between some of the experimental results and more particularly in the conclusions drawn therefrom. The maximum dimension of the particle has been given as 8000 A.[138] from streaming birefringence measurements and as 6500 A.,[67] 6800 A.[144] and 5100–7200 A.[151] from light-scattering measurements on solutions of ionic strength 0.2. There is little information on the shorter dimension, but Schwander[138] gives the value of 10 A. as determined from streaming birefringence. This value is somewhat less than the value of 15–24 A. given by Rowen *et al.*[144] from high-resolution electron microscopy, which is in agreement with the previous value of 15 A. given by Williams[152] and also obtained from measurements with the electron microscope. If these values are of the correct order then the axial ratio will be about 350, which is to be compared with the approximate value of 120 obtained by Cecil and Ogston[60] from sedimentation measurements.

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[147] J. Shack, R. J. Jenkins, and J. M. Thompsett, *J. Biol. Chem.* **198,** 85 (1952).

[148] R. Thomas, *Bull. soc. chim. biol.*, in press.

[149] J. Shack, R. J. Jenkins, and J. M. Thompsett, *J. Biol. Chem.*, **203,** 373 (1953).

[150] P. D. Lawley, Studies in the Behaviour of Polyelectrolytes Doctoral thesis, Nottingham Univ., Nottingham, England, 1953.

The relatively small deformability possessed by the nucleate ion when compared to a typical polyelectrolyte can be attributed to the hydrogen-bonded structure suggested by Gulland *et al.*[48] and to the presence of strong intramolecular Van der Waals forces as postulated by Schwander and Signer.[141] There is also, as has been pointed out by Conway and Butler,[135] an important difference between the nucleate ion and a typical polyelectrolyte, since in the former, the charges ($\rightarrow PO^-$) are carried on the phosphoester "backbone" and in the latter are generally carried on short side chains, thereby permitting greater relative movement of the charged groups.

Comparison of these views on the shape and structure of the molecule with the Crick and Watson structure[49] is interesting. This structure indicates a comparatively rigid molecule in view of the strong hydrogen-bonding and Van der Waals forces between the purine and pyrimidine ring systems of the two chains. The length and rigidity of the molecule will be determined by the equilibrium between the repulsion between the charged groups tending to extend the molecule on the one hand and the hydrogen-bonding and Van der Waals forces tending to contract the molecule on the other. Absorption of sodium ions, with the resulting neutralization of some of the charged groups through ion-pair formation, will disturb this equilibrium and lead to a more compact molecule. The evidence for a rigid non-deformable molecule rests on the independence of the viscosity of infinite dilution on ionic strength as determined from viscosity measurements[134,135] and on the variation of the streaming birefringence in solutions of different viscosity.[140] The latter evidence is capable of different interpretation,[126,142] whereas in the case of the former measurements it is doubtful whether viscosities in very dilute solutions can be determined with sufficient accuracy to decide whether the $\eta_{sp.}/c$—vs.—c curves obtained with and without added electrolyte extrapolate to the same point. It is to be concluded therefore that the deoxypentose nucleate ion in solution is deformable, but to a much lesser extent than a typical polyelectrolyte or uncharged polymer.

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[151] R. F. Steiner, *Trans. Faraday Soc.* **48,** 1185 (1952).

[152] R. C. Williams, *Biochim. et Biophys. Acta* **9,** 237 (1952).

One further fundamental problem should be mentioned. For a deoxypentose nucleate ion having the structure suggested by Crick and Watson[49] and a molecular weight of 8×10^6, the dimensions would be 40,000 A. by 20 A. This length is too great by a factor of five or six and can clearly only be reduced by increasing the smaller dimension either by coiling or by chain-branching. It would therefore appear impossible for the experimental values of 8×10^6 for the molecular weight and 6800 A. by 20 A. for the dimensions to be correct for a particular set of conditions. The value for the molecular weight and length were obtained from the same measurements (light-scattering) but the width is much less certain and depends largely on measurements on the dried material. It is unlikely that a particle of size 40,000 A. by 20 A. should remain as a rigid rod in solution, but would coil to give a much less asymmetric particle as the light-scattering evidence indicates.[67,71] The suggestion[67] that the symmetry is due entirely to chain-branching would appear not to be in agreement with the streaming birefringence results since deformation would not then be possible. It is clearly evident that more reliable experimental data is required before a complete picture of the size and shape of the nucleate ion in solution is obtained.

c. *The Influence of Changes of pH on the Size and Shape of the Deoxypentose Nucleate Ion in Solution*

The comparison of the forward- with the back-titration curve of sodium deoxypentose nucleate (p. 477, Fig. 15) shows that the action of both acid and alkali produces a marked change in the properties of the nucleic acid. This behavior has been ascribed by Gulland *et al.*[48] to an irreversible breaking of the hydrogen bonds existing between the amino and —NH—CO— groups in the nucleate ion. The breaking of the hydrogen bonds occurs at pH values of 5.0 and 11.0, between these values no groups are titrated on the addition of acid or alkali and no hydrogen bonds are broken. In a related study of the viscosity changes produced in a 0.24 % solution of sodium deoxypentose nucleate, Creeth *et al.*[110] observed that the viscosity dropped sharply in the region of the same critical pH values of 5.0 and 11.0 at which the titration of hydrogen-bonded groups occurred. This behavior was interpreted as indicating that the hydrogen bonds unite molecular units which are more symmetrical and of lower molecular weight than the original nucleate. These units become the disperse species in acid and alkaline solution. The earlier suggestion of Vilbrandt and Tennent[111] that the fall in viscosity produced by the action of acid or alkali was due to a depolymerization of the nucleate appears to be untenable in view of the titration evidence[48] that phosphoester bonds are not broken by these reagents.

The solutions studied by Creeth *et al.*[110] were too concentrated to permit any real analysis in terms of molecular dimensions to be made, and more recently Schwander[153] has measured the viscosities of more dilute solutions in 1% sodium chloride solution at pH 3.70 and 6.60 and finds a much smaller decrease of viscosity than that observed by Creeth *et al.* Sedimentation studies by Vilbrandt and Tennent[111] and Cecil and Ogston[60] showed that the action of both acid and alkali causes a decrease in the sedimentation coefficient. Cecil and Ogston found that two separate components appeared after the addition of acid (1 *M* HCl); one was homogeneous and resembled the original nucleate in neutral solution and the other was polydisperse and had apparently been formed by the disaggregation of the original material. The addition of alkali produced a larger lowering of the sedimentation coefficient than did the action of acid and the disaggregation was more complete.

Creeth *et al.*[110] observed that on neutralizing to pH 7.0 a solution of deoxypentose nucleate which had been treated with alkali at pH 12.0, a slow increase of viscosity occurred and after 90 hours the viscosity resembled that of the original solution. This behaviour has been shown by Zamenhof and Chargaff[154] to be due to an artifact having highly thixotropic behaviour. The reversibility of the disaggregation was not observed on treatment with acid.

A very much clearer picture of the changes that occur in sodium deoxypentose nucleate on treatment with dilute acid has been given in a very careful study by Reichmann *et al.*[155] A great criticism of all the previous work is that the pH of solutions was changed by adding small amounts of relatively strong acid. This procedure must inevitably produce transient regions of much lower pH (or higher pH if alkali is added) than that ultimately attained at equilibrium. This treatment will thus produce, in some particles, greater degradation than in the remainder. In order to prevent the deoxypentose nucleate particles from coming into contact with concentrated acid, Reichmann *et al.*[155] adjusted the pH by dialysis. Using the light-scattering method, these authors found that at pH 6.5, 3.0, and 2.6 in 0.2 *M* sodium chloride solution, the deoxypentose nucleate ion has the same molecular weight (7.7×10^6). There is, however, a marked contraction of the ion which is quite pronounced at pH 3.0, but very much greater at pH 2.6. This is shown by the values of the root-mean-square end-to-er distance, which assuming a monodisperse species are: at pH 6.5, 5030 A pH 3.0, 4340 A.; pH 2.6, 2150 A. Furthermore this contraction is con pletely reversible, the value of the end-to-end distance being 5200 A. c neutralizing the solution to pH 6.5 from pH 2.6. It is thus evident that, a pH 2.6 in 0.2 *M* sodium chloride solution, degradation of the particle doe not occur, but only a marked contraction. It is also evident that the de formability of the molecule at pH 2.6 is greater than at pH 6. This will b due to the removal of many of the hydrogen bonds, thus reducing th rigidity of the molecule so that when the charged groups are neutralized b combination with a proton, the molecule will assume the shape approachin that of a random coil. The reversibility of the contraction (which is als reflected in the viscosity measurements of Reichmann *et al.*[155]) is due to th fact that, at pH 2.6 in 0.2 *M* sodium chloride, only about one-half of th amino groups have been titrated and therefore a number of the hydroge bonds will remain unbroken and these are apparently sufficient to hold th macro-ion together. At lower pH values, at this ionic strength, all th hydrogen bonds will be broken and the molecular sub-units, capable c independent existence, produced. Such a change will, in all probability, b irreversible.

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[153] H. Schwander, *Helv. Chim. Acta* **32,** 2510 (1949).

[154] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **186,** 207 (1950).

[155] M. E. Reichmann, B. H. Bunce, and P. Doty, *J. Polymer Sci.* **10,** 109 (1953).

The results of Reichmann *et al.*[155] are not in complete agreement with similar investigation carried out by Horn *et al.*[143] The latter authors foun that an irreversible change in viscosity occurred when the pH of the solution was changed from pH 7.0 to 3.8 in 1 *M* sodium chloride. However, th pH was changed by the addition of 0.01 *M* hydrochloric acid to the solutio and not by dialysis, which may account for the irreversible nature of th change. The higher ionic strength used compared with that employed b Reichmann *et al.* will make the degree of ionization at the different pH values more comparable in view of the influence of ionic strength on th dissociation constants of the amino groups. It would appear important tha much more precise information concerning the degree of ionization is necessary for a true analysis of these studies.

The properties of the products of acid treatment obtained by Horn *et al.*[14 are interesting. Since the change was irreversible, it would appear that th hydrogen-bonded structure had been destroyed and the sub-units, two o more per molecule, liberated. The streaming birefringence results in solutions of different viscosity (adjusted by the addition of glycerol), show tha molecular sub-units are much more deformable than the original nuclei acid and appear to possess a much more typical polyelectrolyte behavio as would be expected for a single, non-hydrogen-bonded polynucleotid chain.

CHAPTER 14

Optical Properties of Nucleic Acids and Their Components

G. H. BEAVEN, E. R. HOLIDAY, AND E. A. JOHNSON

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I. Introduction

Although ultraviolet absorption spectroscopy has been employed for the characterization of nucleic acids and their derivatives for more than twenty years, technical improvements in that period, in particular the introduction of the photoelectric spectrophotometer, have reduced considerably the value of most of the earlier work, which is in consequence not usually referred to here. Similarly, recent advances in instrumentation have stimulated the application of infrared absorption methods to the study of nucleic acids, although the full value of such methods for both analysis and molecular structure determination has not yet been exploited.

In the present field absorption spectroscopy fulfils two principal functions, as an analytical tool and as a basis for deductions concerning structure. To date, the first of these functions has been by far the more important since the hydrolysis products of nucleic acids lend themselves readily to such methods. A large proportion of spectroscopic work in the ultraviolet region has however been carried out with a view to serving an author's own particular analytical requirements, and both the scope of the work and the manner of publication have sometimes reduced its general utility, resulting in much unnecessary duplication. A critical survey of work in this field also demonstrates forcibly that the use of compounds of a very high order of purity does not of itself ensure the production of spectroscopic data of comparable accuracy. The measurement of molecular extinction coefficients to an absolute accuracy of better than 1%, or even 5% (Caster[1]), necessitates careful attention to possible instrumental and operational errors and to inter-instrumental discrepancies. These causes of error have recently been discussed in some detail[2] (Goldring *et al.*[3]).

The data given here are reproduced in accordance with the suggested nomenclature of Brode[4] and Hughes.[5] The following symbols are used:-

$$A = \text{absorbance (optical density)} = \epsilon c d = \log \frac{I_0}{I}$$

where ϵ = molar absorptivity;
c = molar concentration;
d = internal cell length in centimeters;
I_0 = intensity of incident radiation;
I = intensity of transmitted radiation.

Wavelengths (λ) are given in millimicrons (mμ). From the point of view of theoretical interpretation of absorption spectra it would be preferable to tabulate or plot frequencies rather than wavelengths, and if a wide spectral band is to be covered such a plot also gives a more compact spectrum, avoiding excessive spread at longer wavelengths. Since, however, the calibration of all current ultraviolet instruments is given in terms of wavelengths, these are retained here. The manner of presentation of absorption curves has in the past depended on the format favored by the journal in question, which is, with a very few noteworthy exceptions, invariably unsatisfactory from the point of view of the working spectroscopist. All such curves should be given on an adequate scale, have coordinate grids fine enough to permit accurate interpolation in both directions, and if possible be printed in such a way that their scales have a metric basis to permit direct measurement with an ordinary ruler.* It is rarely necessary to plot absorptivities on a logarithmic basis for relatively simple spectra such as those of purines and pyrimidines. The details of shape are thereby blunted and the tedious process of obtaining quantitative estimates from small-scale curves is made even more tiresome and inaccurate. Even if the above conditions are fulfilled it is always desirable to provide in addition a table giving molar absorptivities and wavelengths of principal features, including minima as well as maxima.

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* See pocket on inside back cover for scale drawings of Figs. 3 through 20.

[1] W. O. Caster, *Anal. Chem.* **23,** 1229 (1951).

[2] *Photoelectric Spectrometry Group Bull.*, No. **3** (Oct. 1950).

[3] L. S. Goldring, R. C. Hawes, G. H. Hare, A. O. Beckman, and M. E. Stickney, *Anal. Chem.* **25,** 869 (1953).

[4] W. R. Brode, *J. Opt. Soc. Amer.* **39,** 1022 (1949).

[5] H. K. Hughes, *Anal. Chem.* **24,** 1349 (1952).

II. Bases, Nucleosides, and Mononucleotides

The structural significance of the ultraviolet absorption spectra of purines and pyrimidines has been well reviewed by Jordan,[6] who, following Marshall and Walker,[7] has emphasized the role played by ionization in the spectral changes observed on varying the pH of solutions. Marshall and Walker have pointed out that the more accessible pyrimidines, including those concerned here, have a number of functional groups as substituents which make detailed interpretation of their spectra very difficult. These workers, and also Boarland and McOmie[8] and Brown and Short,[9] give spectra for many simple pyrimidines, including pyrimidine itself. The ultraviolet absorption spectra of the simple diazines have been discussed by Halverson and Hirt,[10] who, however, employ the older spectrum of pyrimidine determined by Heyroth and Loofbourow.[11] Less attention has been devoted to a general examination of the purines. Here again, however, Stimson and Reuther[12] and Cavalieri *et al.*[13] have attributed the spectral changes observed with change in pH to keto–enol tautomerism, although the close coincidence of pH values determined by titration with the pH regions where such changes occur, points unmistakably to their ionic character. The importance of pH control when measuring purine absorption spectra was emphasised as early as 1930 by one of us (E. R. H.[14]), but measurements are still frequently made at an arbitrary pH value which lies so close to a pK that a mixture of ionic species is present. Spectroscopic measurements have in fact proved a useful method for the determination of many additional pK values, some of which lie outside the range of convenient measurement by conventional means. The work of Shugar and

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[6] D. O. Jordan, *Progr. Biophys. and Biophys. Chem.* **2,** 51 (1951); *Ann. Rev. Biochem.* **21,** 209 (1952).

[7] J. R. Marshall and J. Walker, *J. Chem. Soc.* **1951,** 1004.

[8] M. P. V. Boarland and J. F. W. McOmie, *J. Chem. Soc.* **1952,** 3716, 3722, 4942.

[9] D. J. Brown and L. N. Short, *J. Chem. Soc.* **1953,** 331.

[10] F. Halverson and R. C. Hirt, *J. Chem. Phys.* **19,** 711 (1951).

[11] F. F. Heyroth and J. R. Loofbourow, *J. Am. Chem. Soc.* **56,** 1728 (1934).

[12] M. M. Stimson and M. A. Reuther, *J. Am. Chem. Soc.* **65,** 153 (1943).

[13] L. F. Cavalieri, A. Bendich, J. F. Tinker, and G. B. Brown, *J. Am. Chem. Soc.* **70,** 3875 (1948).

[14] E. R. Holiday, *Biochem. J.* **24,** 619 (1930).

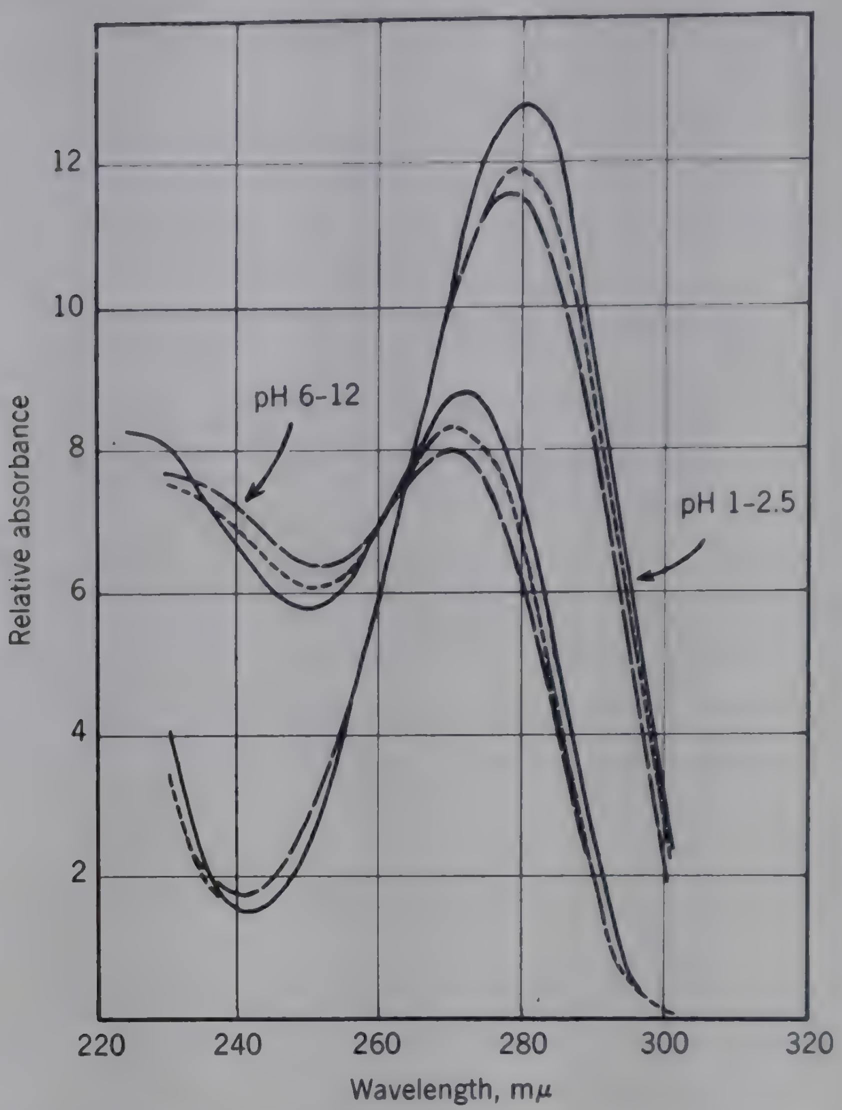

FIG. 1. Isomeric cytidylic acids (Cohn, unpublished).
———— 5′- and deoxy-
- - - - - - 3′-
—— —— 2′-

Fox[15,16] is a good illustration of the application of such methods, and is noteworthy for the excellent style of publication of the spectral data.

The absorption characteristics of a given compound in aqueous solution can therefore be given as a series of spectra of the individual ionic species, together with a list of the corresponding pK values preferably as determined spectroscopically.

The pK value for a given ionization can be determined from the following expression (see Hammett and co-workers[17] and Edwards[18]):

$$pK_a = \text{pH} - \log \frac{\epsilon_{HA} - \epsilon}{\epsilon - \epsilon_A}$$

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[15] D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta* **9**, 199 (1952).
[16] J. J. Fox and D. Shugar, *Biochim. et Biophys. Acta* **9**, 369 (1952).
[17] L. A. Flexser, L. P. Hammett, and A. Dingwall, *J. Am. Chem. Soc.* **57**, 2103 (1935).
[18] L. J. Edwards, *Trans. Faraday Soc.* **46**, 723 (1950).

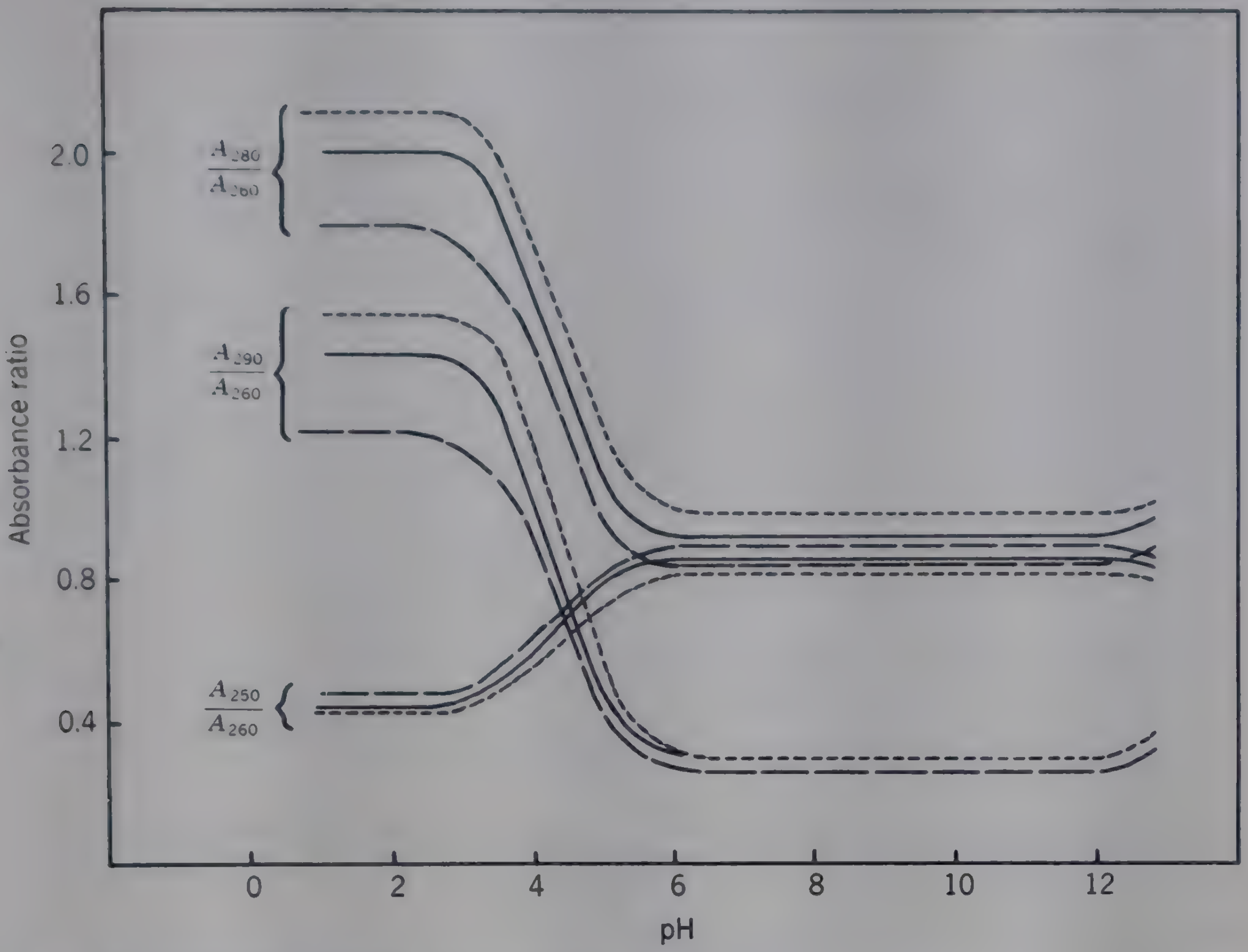

FIG. 2. Relation between absorbance ratios and pH for the isomeric cytidylic acids (Cohn, unpublished).

- - - - - - 5′- and deoxy-
——— 3′-
—— —— 2′-

where ϵ_{HA} and ϵ_{A} are the absorptivities of the two ionic species, and ϵ is the absorptivity determined at the given pH. The thermodynamic value of pK_a may be obtained by appropriate correction for the ionic strengths.

The tables given here also include absorbance ratios based largely on the very comprehensive work of Cohn[19] (with subsequent additions and revisions). These have considerable utility for the identification of compounds in solutions of unknown concentration, but their precise values will be critically dependent on the wavelength calibration of the spectrophotometer, and variations must be expected when different instruments are used. The relationships of these ratios to pH values and to the absorption spectra are illustrated for the isomeric cytidylic acids by Figs. 1 and 2 (Cohn, unpublished). It may be noted that the phosphate ionizations appear to be without significant effect on the spectra, but this is not necessarily always true (see Table III).

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[19] E. Volkin and W. E. Cohn, Methods of Biochemical Analysis, Vol. 1, p. 287, Interscience, New York, 1954.

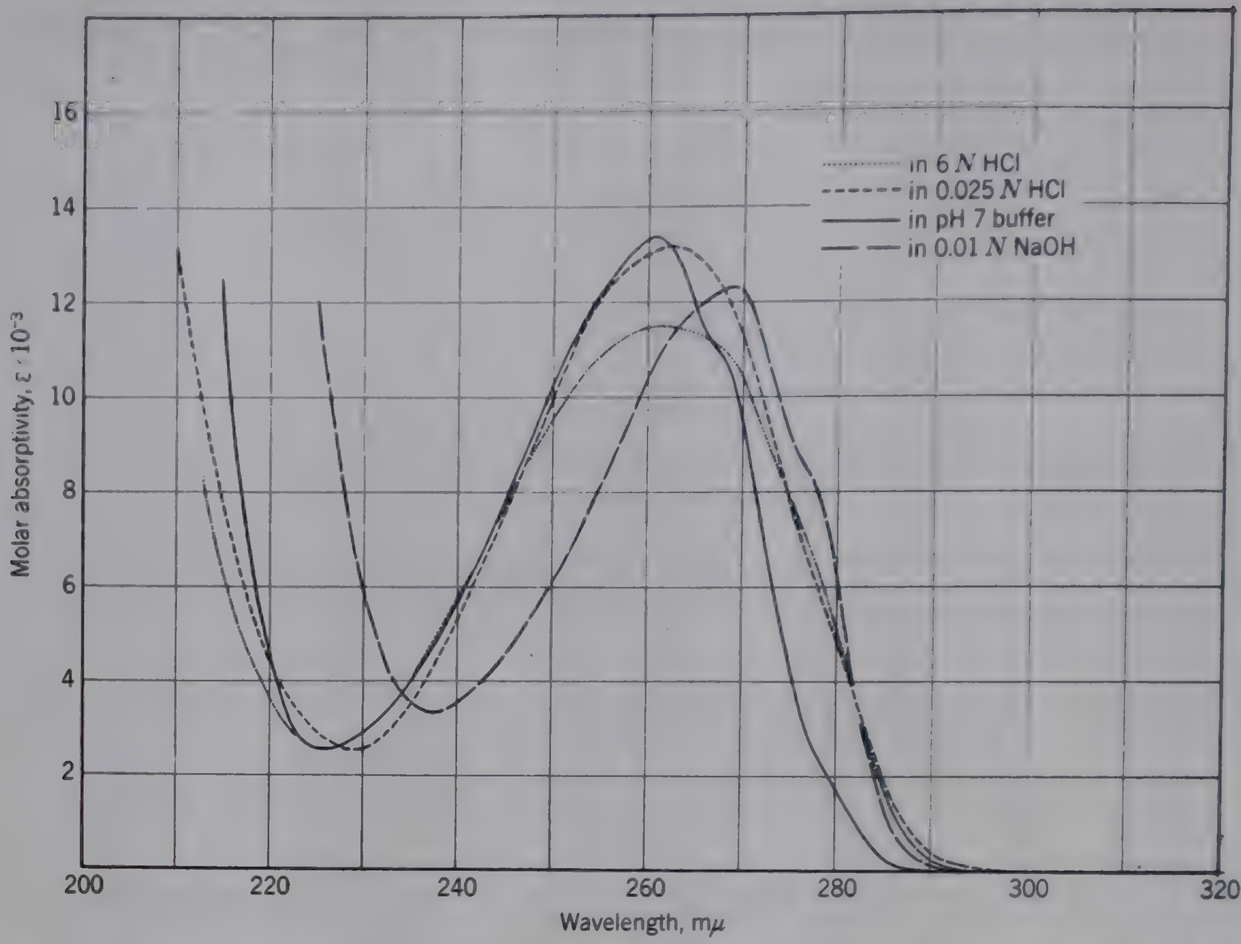

FIG. 3. Adenine (Johnson, unpublished). The absorption spectra illustarted here and in Figs. 4–20 will be found drawn to scale in the pocket on the inside back cover of the volume.

From such data it is possible to select wavelengths and obtain coefficients for the identification and quantitative estimation of bases, nucleosides, and nucleotides obtained from nucleic acids by any of the methods described in other chapters.

1. BASES

In spite of the fact that ultraviolet absorption data for adenine have been reported in at least thirteen separate papers, no set of data has yet been published which can be considered in any way comprehensive. This is equally true of the other purines found in or derived from nucleic acids. It has therefore seemed necessary to undertake a more thorough examination of them. Spectra for adenine, hypoxanthine, guanine, and xanthine (measured with a calibrated Unicam SP 500 spectrophotometer, using specimens all purified by various methods in this laboratory) are given in Figs. 3 to 6, respectively, and corresponding numerical data in Table I.

Spectral data for adenine and guanine in dilute acid and in some cases at other pH values have been determined recently by Hotchkiss,[20] Cavalieri *et al.*,[13] Vischer and Chargaff,[21] Kerr *et al.*,[22] Tsuboi and Stowell,[23]

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[20] R. D. Hotchkiss, *J. Biol. Chem.* **175,** 315 (1948).

[21] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176,** 703 (1948).

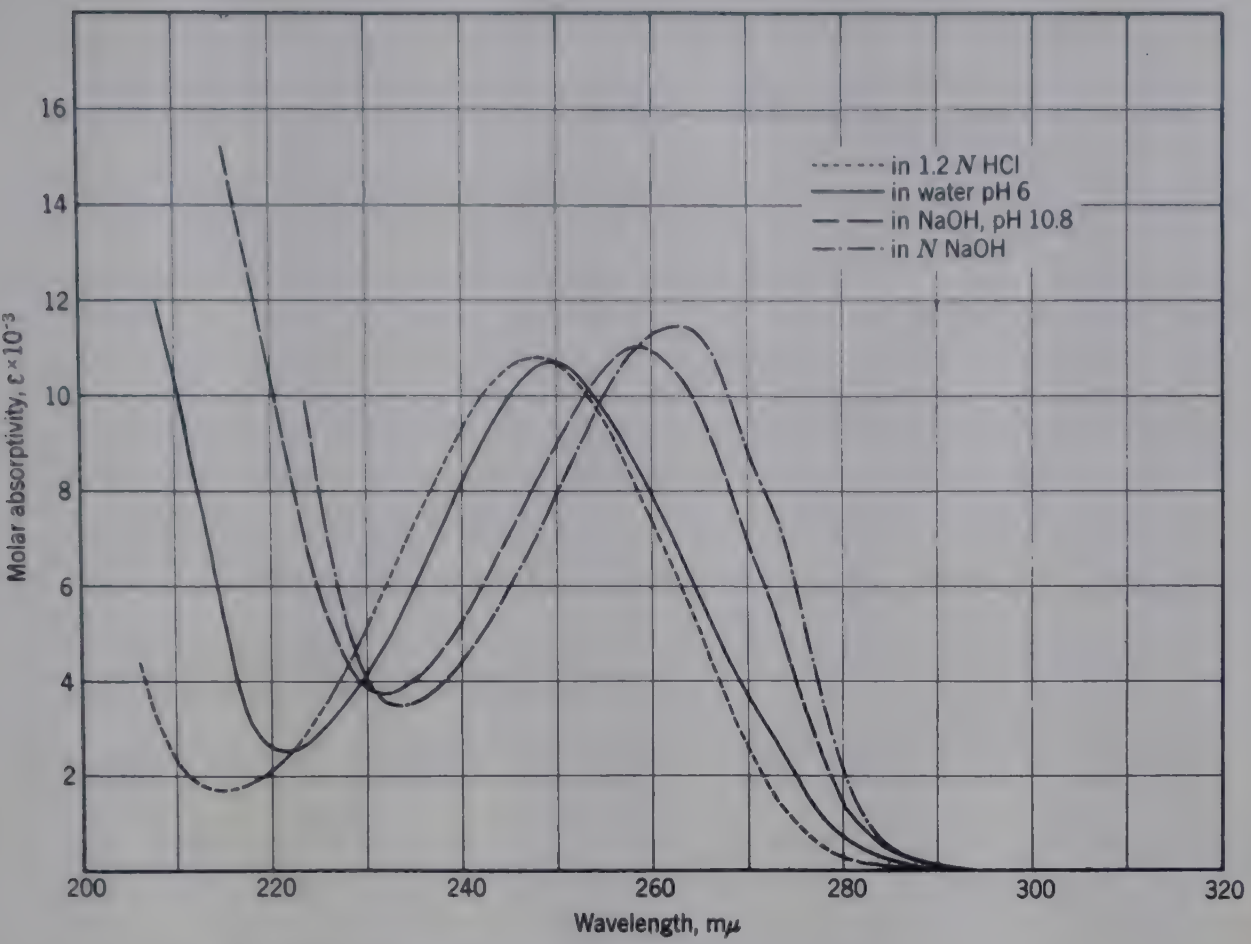

FIG. 4. Hypoxanthine (Johnson, unpublished).

Wyatt,[24] Markham and Smith,[25] and Loring *et al.*;[26] and the results presented here agree quite well with the more recent of these. It does not seem to have been observed previously, however, that both compounds have an additional p*K* in the region of pH 0, and show marked spectral changes presumably associated with the ionization of one of the ring nitrogen atoms. Some methods of spectroscopic analysis have failed to take this into account.

The first two references of this group also contain data for hypoxanthine and xanthine, absorption curves for which have in addition been published by Stimson and Reuther.[12]

Absorption data for cytosine, uracil, and thymine have been published in recent years by Hotchkiss,[20] Vischer and Chargaff,[21] Stimson,[27] Wyatt,[24] and Shugar and Fox.[15] Excellent data for the first two of these are given by

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[22] S. E. Kerr, K. Seraidarian, and M. Wargon, *J. Biol. Chem.* **181,** 761 (1949).

[23] K. K. Tsuboi and R. E. Stowell, *Biochim. et Biophys. Acta* **6,** 192 (1950).

[24] G. R. Wyatt, *Biochem. J.* **48,** 584 (1951).

[25] R. Markham and J. D. Smith, *Biochem. J.* **49,** 401 (1951).

[26] H. S. Loring, J. L. Fairley, H. W. Bortner, and H. L. Seagran, *J. Biol. Chem.* **197,** 809 (1952).

[27] M. M. Stimson, *J. Am. Chem. Soc.* **71,** 1470 (1949).

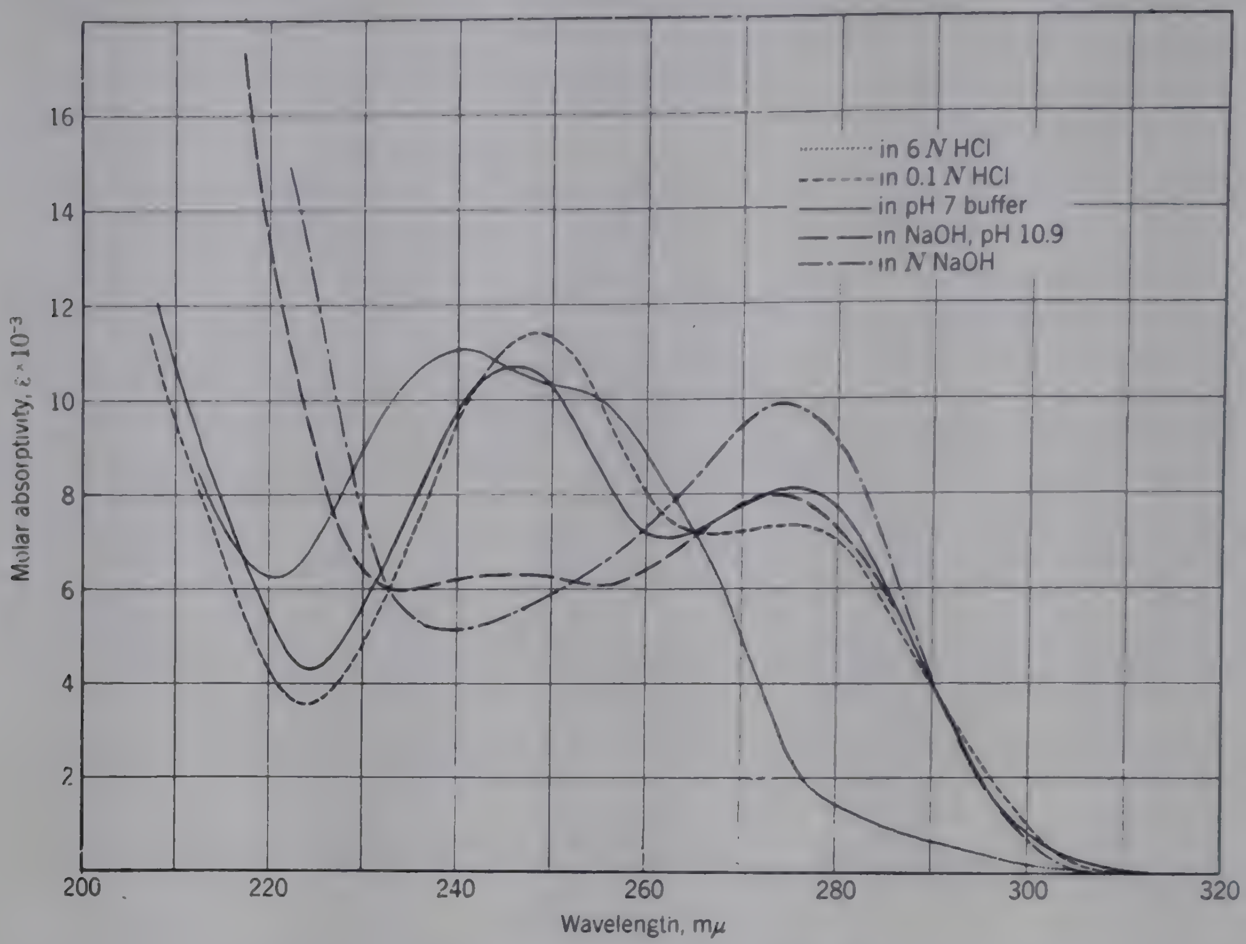

FIG. 5. Guanine (Johnson, unpublished).

Ploeser and Loring,[28] and Andrisano and Modena[29] discuss the spectra o numerous pyrimidines including uracil, at various pH values, withou mention of a pK. Wyatt[30] and Cohn[31] first gave data for 5-methylcytosine and Wyatt and Cohen[32] have reported 5-hydroxymethylcytosine isolate from bacteriophage nucleic acids, with maxima at 279 mμ, ϵ = 9700, i 0.1 N hydrochloric acid, at 269.5 mμ in pH 7.4 buffer, and at 283.5 mμ i 0.1 N sodium hydroxide. For all except this last compound very complet data have been given by Shugar and Fox,[15] and the curves published her (Figs. 7 to 10) have been re-drawn from their paper. It has been pointe out by Cohn (private communication) that uracil has a pK in the region o pH 0.5; the spectrum in 6 N HC1 was therefore determined in this labora tory and added to those of the other ionic species.

Good general agreement was found between the data for the pyrimidine by Shugar and Fox, those given here for the purines, and an extensive serie

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[28] J. M. Ploeser and H. S. Loring, *J. Biol. Chem.* **178,** 431 (1949).

[29] R. Andrisano and G. Modena, *Gazz. chim. ital.* **81,** 405 (1951).

[30] G. R. Wyatt, *Biochem. J.* **48,** 581 (1951).

[31] W. E. Cohn, *J. Am. Chem. Soc.* **73,** 1539 (1951).

[32] G. R. Wyatt and S. S. Cohen, *Nature* **170,** 1072 (1952).

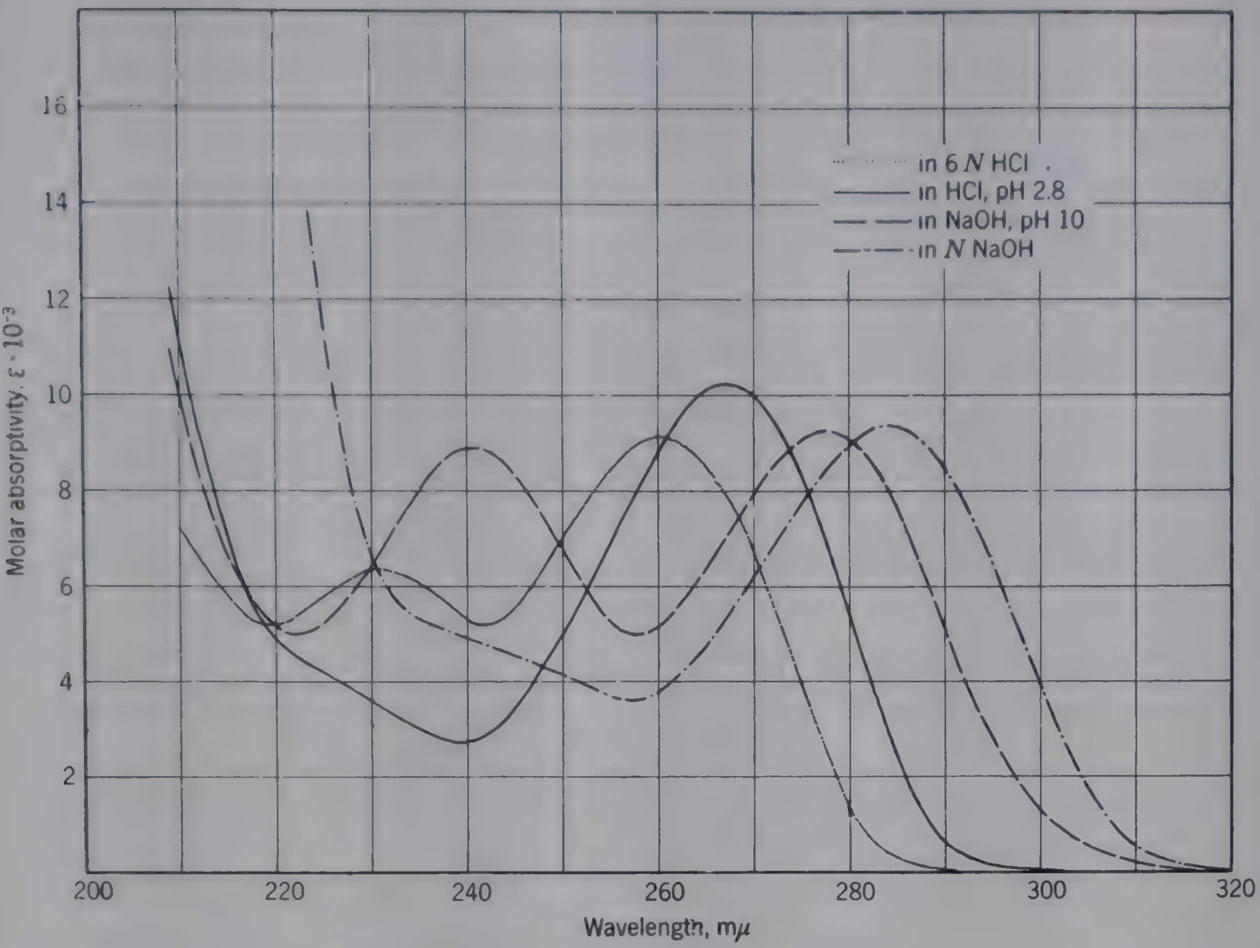

FIG. 6. Xanthine (Johnson, unpublished).

of absorbance ratios and values for ϵ at 260 mμ determined by Cohn[19] (with subsequent revisions and additions), whose complete spectra were unfortunately not available. In view of the limitations to the accuracy of absorbance ratios mentioned above, in Table I the values of Cohn are quoted for the sake of consistency whenever they are available, supplemented from other sources when necessary. Some variations from values obtained by inspection of the absorption curves will therefore be found. Table I is intended to give data for individual ionic species, and the p*K* values in the second column are placed between the two species to whose interconversion they apply. Wherever possible the determinations for each species were carried out at a pH removed at least 1.5 and preferably 2 units from the nearest p*K*, and in solutions of low ionic strength.

The determination of absorption spectra of purines and pyrimidines at low temperatures has been carried out on sublimed films at 90° K. by Brown and Randall,[33] and on sublimed films and also solutions in mixed organic solvents which form glasses at 77° K. and 23° K. by Sinsheimer *et al.*[34] The vibrational fine-structure is enhanced, particularly in pyrimidines such

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[33] G. L. Brown and J. T. Randall, *Nature* **163,** 209 (1949).

[34] R. L. Sinsheimer, J. F. Scott, and J. R. Loofbourow, *J. Biol. Chem.* **187,** 299, 313 (1950); *J. Am. Chem. Soc.* **74,** 275 (1952).

TABLE I
BASES

Compound	pK[a]	Maxima and minima[b]								ϵ_{260}	Absorbance ratios[c]		
		$\lambda_{max.1}$	$\epsilon_{max.1}$	$\lambda_{min.1}$	$\epsilon_{min.1}$	$\lambda_{max.2}$	$\epsilon_{max.2}$	$\lambda_{min.2}$	$\epsilon_{min.2}$		$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$
Adenine[d]	<0[e]	—	—	—	—	—	—	—	—	$\overline{<}11.5$	—	—	—
	4.1	262.5	13.15	229	2.55	—	—	—	—	13.0	0.76	0.375	0.035
	9.8	260.5	13.35	226	2.55	—	—	—	—	13.3	0.76	0.125	0.005
		269	12.3	237	3.35	—	—	—	—	10.45	0.57	0.60	0.025
Hypoxanthine[d]	ca. 2	248	10.8	215	1.7	—	—	—	—	7.35	1.45[e]	0.04[e]	0[e]
	ca. 9	249.5	10.7	221.5	2.55	—	—	—	—	7.9	1.32	0.092	0.010
	>12[e]	258.5	11.05	232	3.75	—	—	—	—	10.95	0.84	0.124	0.007[e]
		262.5	11.45	233.5	3.5	—	—	—	—	11.3	0.705[e]	0.19[e]	0.007[e]
Guanine[d]	<0[e]	—	—	—	—	—	—	—	—	$\overline{>}8.8$	—	—	—
	3.2	275.5	7.35	267	7.15	248.5	11.4	224	3.55	8.0	1.37	0.84	0.495
	9.6	275.5	8.15	262	7.05	246	10.7	224.5	4.3	7.2	1.42	1.04	0.54
	ca. 12.5[e]	273.5	8.0	255	6.05	246	6.3	234	6.0	6.4	0.985[e]	1.135[e]	0.585[e]
		274	9.9	239.5	5.15	—	—	—	—	7.3	0.805[e]	1.24[e]	0.605[e]
Xanthine[d]	ca. 0.8	260	9.15	242	5.2	230.5	6.35	219.5	5.2	9.15	0.765[e]	0.15[e]	0.005[e]
	7.8	267	10.25	239.5	2.7	—	—	—	—	8.85	0.565[e]	0.61[e]	0.07[e]
	12	277.5	9.3	257.5	5.0	240.5	8.9	222	5.0	5.2	1.29	1.71	0.92
		284	9.4	257	3.6	—	—	—	—	3.75	1.11[e]	2.39[e]	2.27[e]
Cytosine[f]	4.5	276	10.0	238.5	1.2	210	9.7	—	—	6.0	0.48	1.53	0.78
	12.2	267	6.13	247	4.3	—	—	—	—	5.55	0.78	0.58	0.08
		282	7.86	250.5	1.4	—	—	—	—	2.35	0.595[g]	3.28[g]	2.6[g]

5-Methylcytosine[f]	4.8 12.4	283.5	9.79	242	0.9	210.5	12.0	—	—	3.6	0.41	2.66	2.42
		273.5	6.23	251.5	3.6	210.5	14.2	—	—	4.45	0.81	1.20	0.55
		289.5	8.05	253.5	1.35	—	—	—	—	1.7	0.85[g]	3.75[g]	4.75[g]
Thymine[f]	ca. 0 9.9 >13	—	—	—	—	—	—	—	—	—	—	—	—
		264.5	7.89	233.5	1.9	207	9.5	—	—	7.4	0.67	0.53	0.09
		291	5.44	244	2.2	—	—	—	—	3.7	0.65	1.31	1.41
		—	—	—	—	—	—	—	—	$\lessgtr$3.6	—	—	—
Uracil[f]	ca. 0.5 9.5 >13	260[e]	7.80[e]	228.5[e]	1.52[e]	—	—	—	—	7.8[e]	0.795[e]	0.30[e]	0.05[e]
		259.5	8.20	227.5	1.8	—	—	—	—	8.2	0.84	0.175	0.01
		284	6.15	241.5	2.15	—	—	—	—	4.1	0.71	1.40	1.27
		—	—	—	—	—	—	—	—	4.1	—	—	—

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[a] For references see Chapters 6 and 13.

[b] Wavelengths in mμ, $\epsilon \times 10^{-3}$

[c] Wherever available, data from Cohn.[19]

[d] Data for maxima, minima, and ϵ_{260} from Johnson (unpublished).

[e] Johnson (unpublished).

[f] Data for maxima, minima, and ϵ_{260} from Shugar and Fox.[15]

[g] Shugar and Fox.[15]

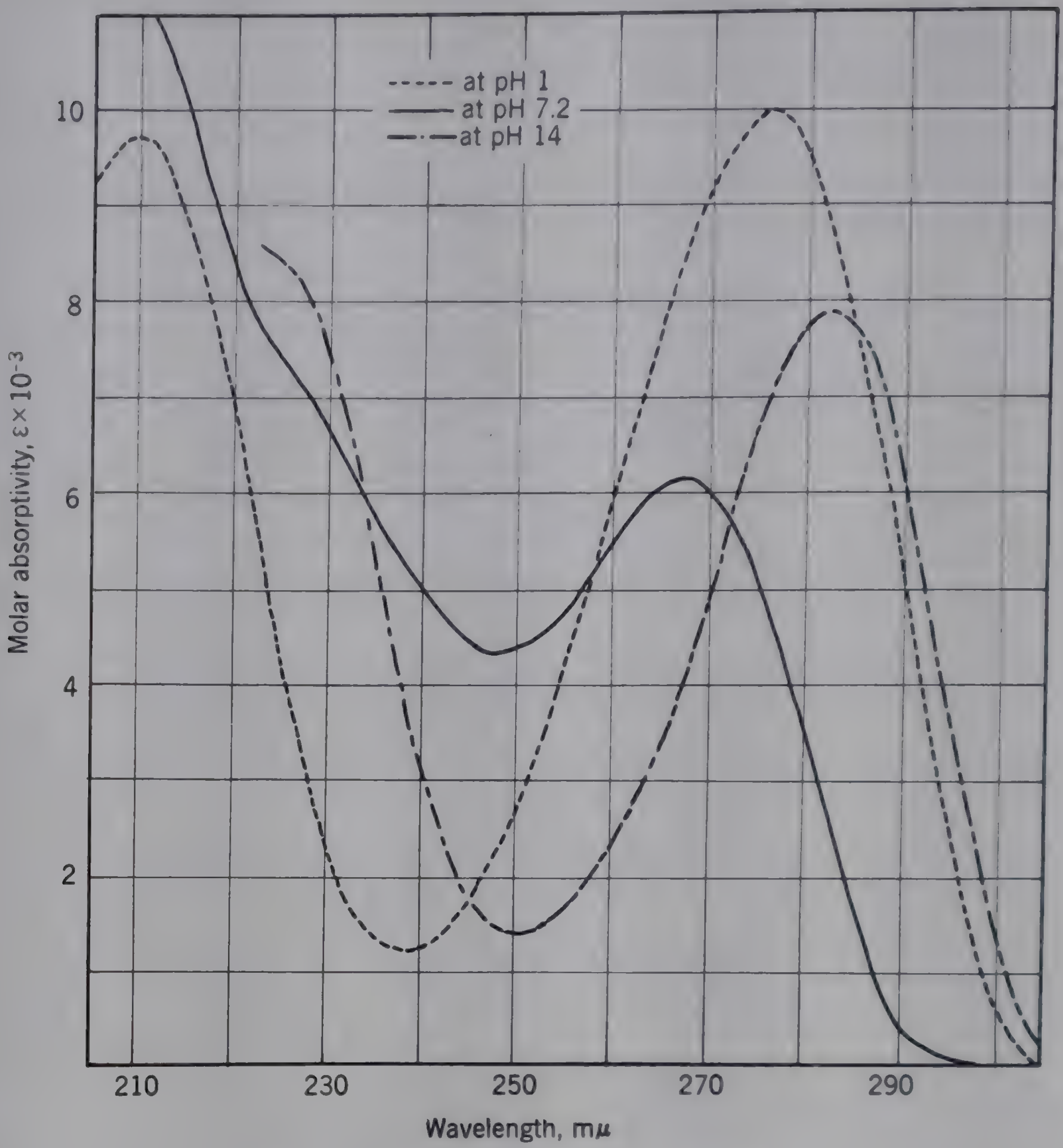

Fig. 7. Cytosine (Shugar and Fox[15]).

as thymine, and to a much greater extent in sublimed films than in glasses. Changes with aging of sublimed films are observed which suggest that these are initially largely amorphous but slowly become crystalline, a process accelerated by the presence of water vapor.

2. Nucleosides

Since ribosides may be obtained by hydrolysis of PNA with much greater ease than the deoxy- derivatives from DNA, the greater part of the available spectroscopic data refers to the former. A good series of absorption curves for deoxyribosides has been published by MacNutt,[35] who examined these derivatives of guanine, hypoxanthine, thymine, cytosine, and uracil. Manson and Lampen[36] give absorption curves for the deoxyribosides of

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[35] W. S. MacNutt, *Biochem. J.* **50**, 384 (1952).

[36] L. A. Manson and J. O. Lampen, *J. Biol. Chem.* **191**, 87 (1951).

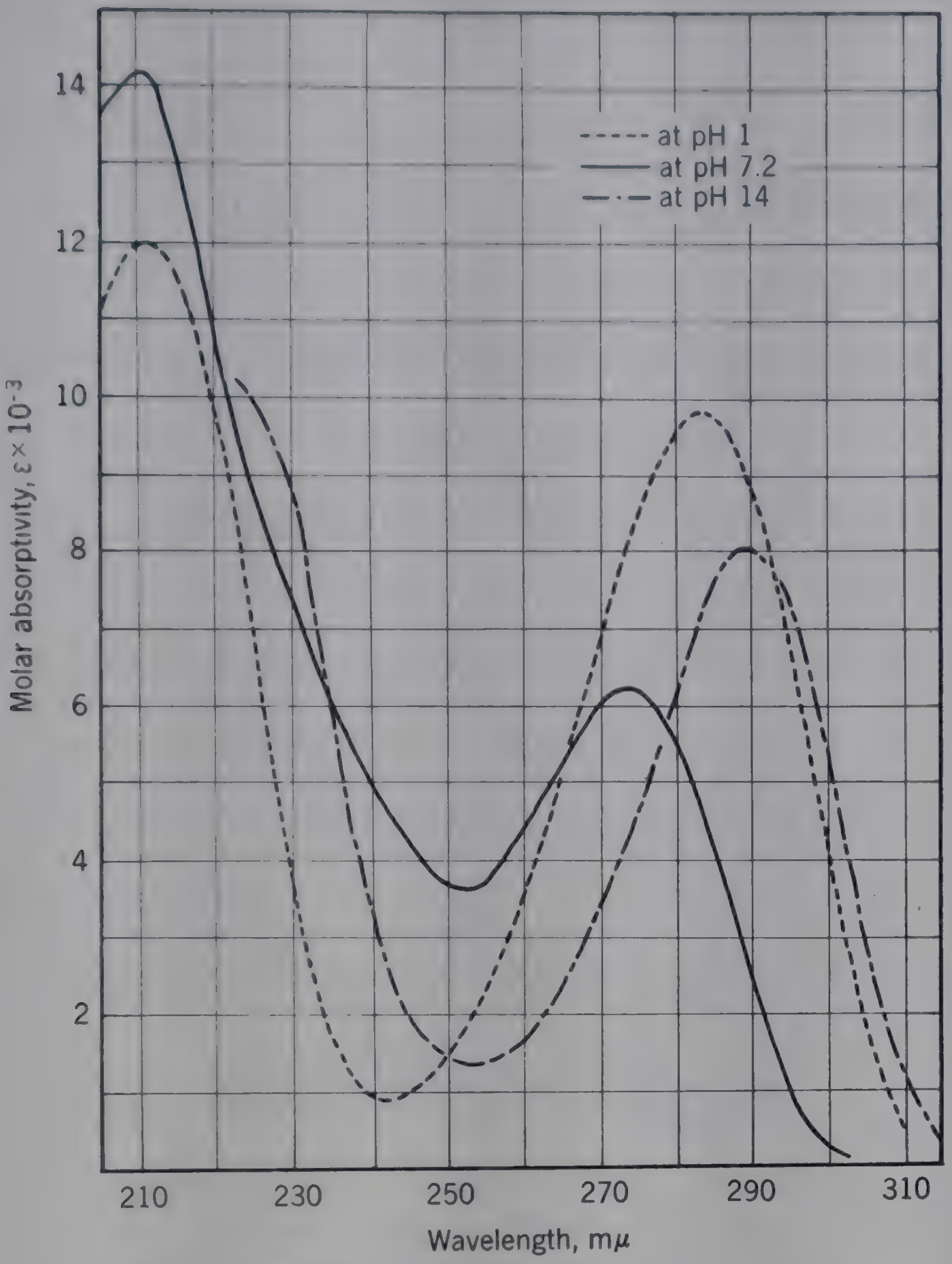

FIG. 8. 5-Methylcytosine (Shugar and Fox[15]).

hypoxanthine, thymine, and cytosine at neutral pH, and Gulland and Story[37] compared guanosine and guanine deoxyriboside. For purine ribosides Hotchkiss[20] gives data for adenosine and guanosine, although in a form quite unsuitable for general use without extensive arithmetical conversion, and Kalckar[38] includes data for these two and for inosine. The differences between ribo- and the corresponding deoxyribo- derivatives may be expected to be very small (compare curves given by MacNutt[35] for guanine and hypoxanthine deoxyribosides with Figs. 12 and 13), but Fox and Shugar[16] have shown that, at least for pyrimidine derivatives, real distinctions can be detected. The realization of the precision obtainable by the use of photoelectric spectrophotometers has come very recently in some quarters, since

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[37] J. M. Gulland and L. F. Story, *J. Chem. Soc.* **1938,** 692.
[38] H. M. Kalckar, *J. Biol. Chem.* **167,** 429, 445 (1947).

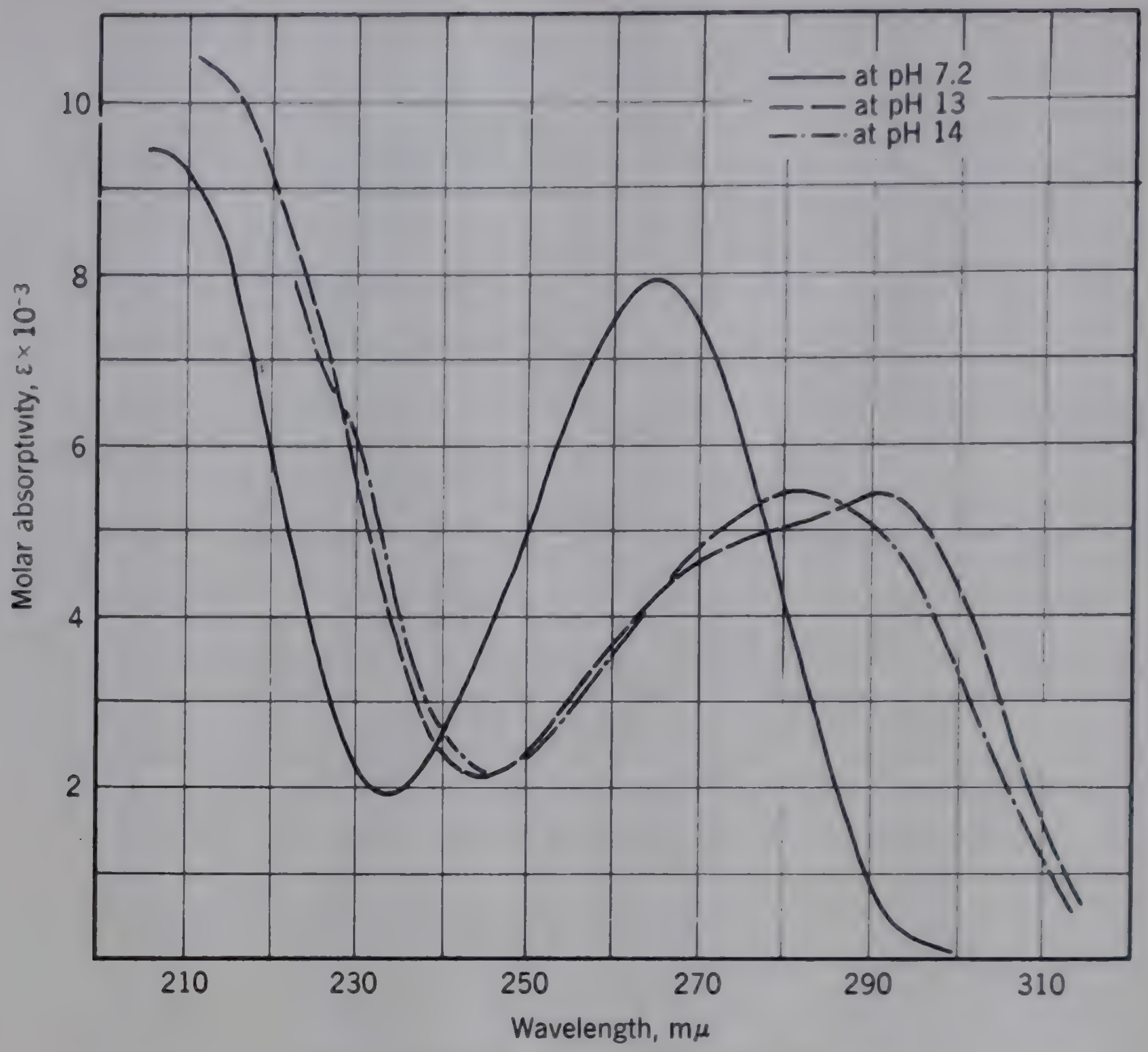

FIG. 9. Thymine (Shugar and Fox[15]).

Kalckar[38] was apparently unable to distinguish adenosine from adenine even though using such an instrument. Schlenk and co-workers[39] give an absorption curve for xanthosine at pH 7.5, and curves for synthetic xanthosine are given by Todd and co-workers.[40] Even allowing for unsuitable pH values the latter are in poor agreement with the results of Schlenk and those given here, and also with the much older values of Gulland et al.,[41] all of which are reasonably consistent.

The incompleteness of the data available for purine ribosides seemed again to require a more systematic investigation, the results of which are given in Figs. 11 to 14, and in Table II. For the pyrimidine nucleosides recourse was had to Fox and Shugar[16] (Figs. 15 to 18), again by far the most complete set of data, and for 5-methylcytosine deoxyriboside to Dekker and Elmore.[42] Absorbance ratios are from Cohn wherever available. Other

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[39] M. L. Schaedel, M. J. Waldvogel, and F. Schlenk, *J. Biol. Chem.* **171,** 135 (1947).

[40] G. A. Howard, A. C. McLean, G. T. Newbold, F. S. Spring, and A. R. Todd, *J. Chem. Soc.* **1949,** 232.

[41] J. M. Gulland, E. R. Holiday, and T. F. Macrae, *J. Chem. Soc.* **1934,** 1639.

[42] C. A. Dekker and D. T. Elmore, *J. Chem. Soc.* **1951,** 2864.

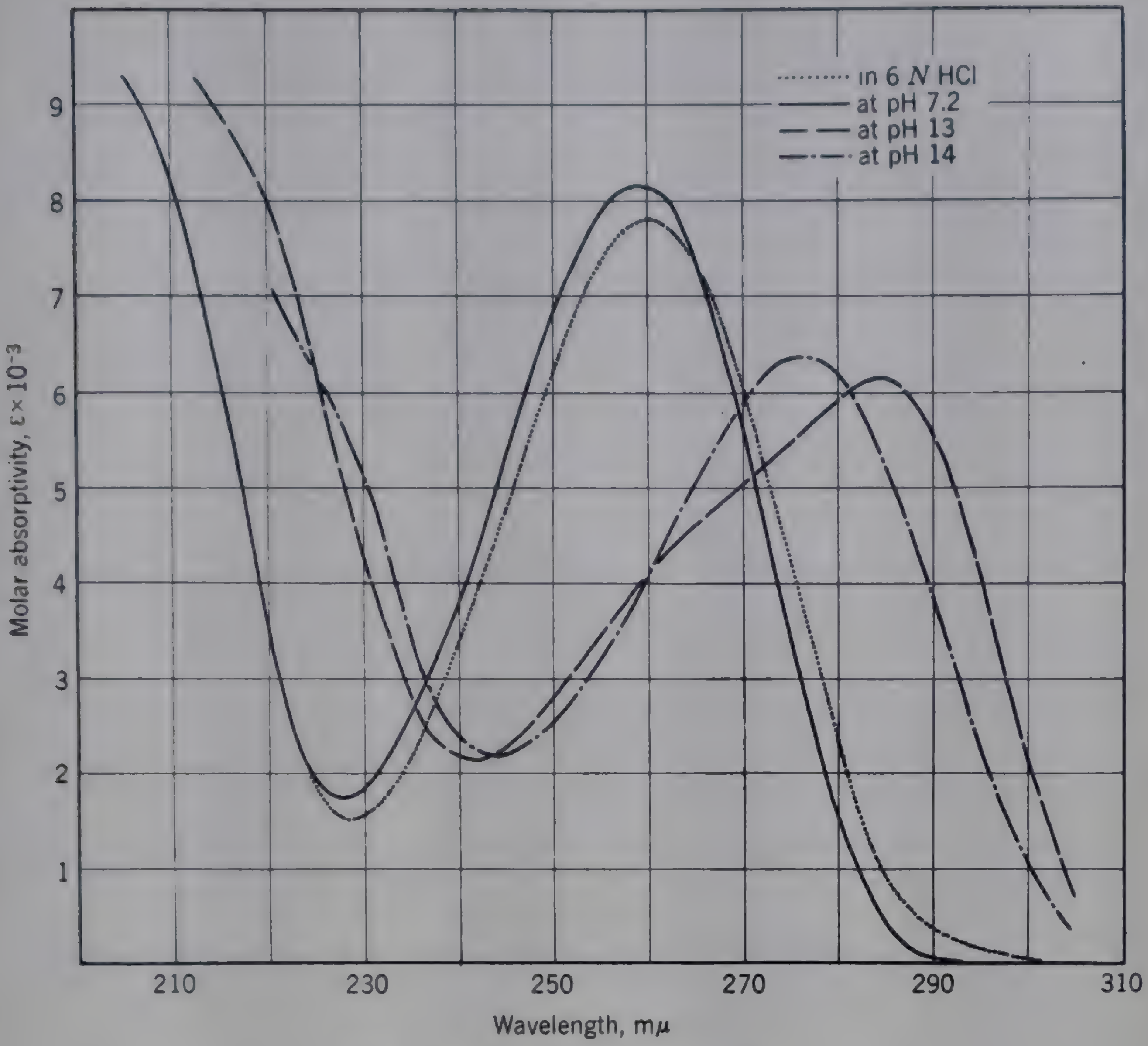

FIG. 10. Uracil (Shugar and Fox[15]).

data on pyrimidine nucleosides have been published by Hotchkiss,[20] Loring et al.,[43] and Lampen and co-workers.[44]

The ionization in the pH 12–13 region observed in the free pyrimidine bases is probably attributable to the acidic dissociation of the 2-hydroxyl group, and in the purines to the acidic dissociation of the iminazole NH group. In the nucleosides both of these are blocked, and the spectroscopic changes in the same pH region can in this case be attributed to a sugar hydroxyl dissociation. (See Jordan[6] and Shugar and Fox[15,16].)

The first demonstration that the sugar group in the naturally occurring nucleosides was attached to the purine nucleus in the 9-position rather than in the 7- was carried

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[43] H. S. Loring, H. W. Bortner, L. W. Levy, and M. L. Hammell, *J. Biol. Chem.* **196,** 807 (1952).

[44] T. P. Wang, H. Z. Sable, and J. O. Lampen, *J. Biol. Chem.* **184,** 17 (1950).

TABLE II

Nucleosides

Compound	pK[a]	Maxima and minima[b]								ϵ_{260}	Absorbance ratios[c]		
		$\lambda_{max.1}$	$\epsilon_{max.1}$	$\lambda_{min.1}$	$\epsilon_{min.1}$	$\lambda_{max.2}$	$\epsilon_{max.2}$	$\lambda_{min.2}$	$\epsilon_{min.2}$		$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$
Adenosine[d]	3.4	257	14.6	230	3.5	—	—	—	—	14.3	0.84	0.215	0.03
	13	259.5	14.9	227	2.25	—	—	—	—	14.9	0.78	0.144	0.002
		—	—	—	—	—	—	—	—	—	—	—	—
Inosine[d]	1.2	251	10.9	221	2.45	—	—	—	—	9.0	1.21[e]	0.11[e]	0[e]
	8.7	248.5	12.25	223	3.4	—	—	—	—	7.1	1.68	0.25	0.025
		253	13.1	224.5	2.55	—	—	—	—	11.7	1.05	0.18	0.008[e]
Guanosine[d]	2.2	256.5	12.2	228	2.4	—	—	—	—	11.75	0.94[e]	0.695[e]	0.50[e]
	9.5	252.5	13.65	223.5	2.85	—	—	—	—	11.7	1.15	0.67	0.275
		258–266	11.3	231	4.1	—	—	—	—	11.3	0.89	0.61	0.13
Xanthosine[d]	*ca.* 0	—	—	—	—	—	—	—	—	—	—	—	—
	5.5	263	8.95	248	6.4	235	8.4	217	3.9	8.7	0.75	0.28	0.03
	ca. 13[e]	278	8.9	264.5	7.05	248.5	10.2	222.5	2.75	7.65	1.30	1.13	0.61
		276	9.3	262	7.5	251.5	8.6	230	4.5	7.65	1.12	1.16	0.59
Cytidine[f]	4.1	280	13.4	241.5	1.7	212.5	10.1	—	—	6.4	0.45	2.10	1.55
	ca. 13	271	9.1	250.5	6.5	229.5	8.3	226	8.2	7.55	0.86	0.93	0.28
		273	9.2	251.5	6.03	—	—	—	—	7.0	0.865[g]	1.17[g]	0.56[g]
Cytosine deoxyriboside[f]	4.3	280	13.2	241	1.5	212.5	10.2	—	—	6.15	0.42[g]	2.15[g]	1.61[g]
	ca. 13	271	9.0	250	6.13	—	—	—	—	7.35	0.83[g]	0.965[g]	0.305[g]
		272.5	9.28	248.5	5.70	—	—	—	—	7.05	0.81[g]	1.09[g]	0.405[g]

5-Methylcytosine deoxyriboside[h]	*ca.* 4.5	286	11.61	245	1.05	209	9.78	—	—	3.2	0.45	3.4	3.55
	ca. 13	277	8.81	255	5.43	206	13.38	—	—	6.0	0.95	1.45	0.93
		—	—	—	—	—	—	—	—	—	—	—	—
Thymidine[f]	9.8	267	9.65	235	2.25	207.5	9.55	—	—	8.75	0.65	0.72	0.235
	>13	267	7.38	240.5	4.58	—	—	—	—	6.65	0.75	0.67	0.16
		—	—	—	—	—	—	—	—	$\bar{<}$6.5	—	—	—
Uridine[f]	9.25	262	10.1	230.5	2.05	—	—	—	—	9.95	0.74	0.35	0.03
	>13	262	8.5	236.5	4.48	—	—	—	—	7.35	0.83	0.29	0.02
		—	—	—	—	—	—	—	—	$\bar{<}$7.1	—	—	—
Uracil deoxyriboside[f]	9.3	262	10.2	231	2.2	—	—	—	—	10.1	0.72[g]	0.375[g]	—
	>13	262	7.63	242	5.35	—	—	—	—	7.55	0.81[g]	0.31[g]	—
		—	—	—	—	—	—	—	—	$\bar{>}$7.65	—	—	—

[a] For references see Chapters 6 and 13.

[b] Wavelengths in mμ, $\epsilon \times 10^{-3}$

[c] Wherever available, data from Cohn.[19]

[d] Data for maxima, minima, and ϵ_{260} from Johnson (unpublished).

[e] Johnson (unpublished).

[f] Data for maxima, minima, and ϵ_{260} from Fox and Shugar.[16]

[g] Fox and Shugar.[16]

[h] All data from Dekker and Elmore,[42] but see Fox and Shugar.[16]

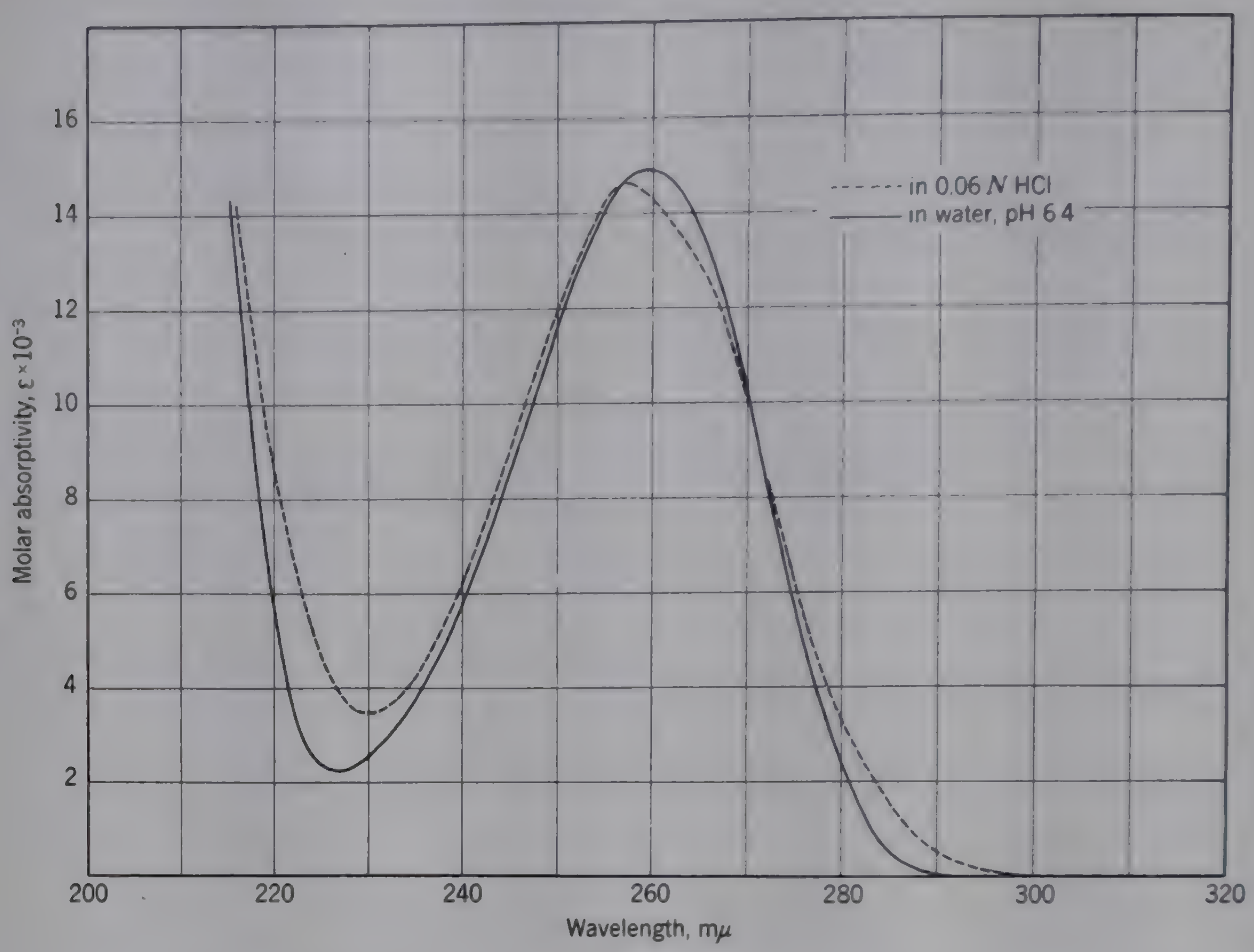

FIG. 11. Adenosine (Johnson, unpublished).

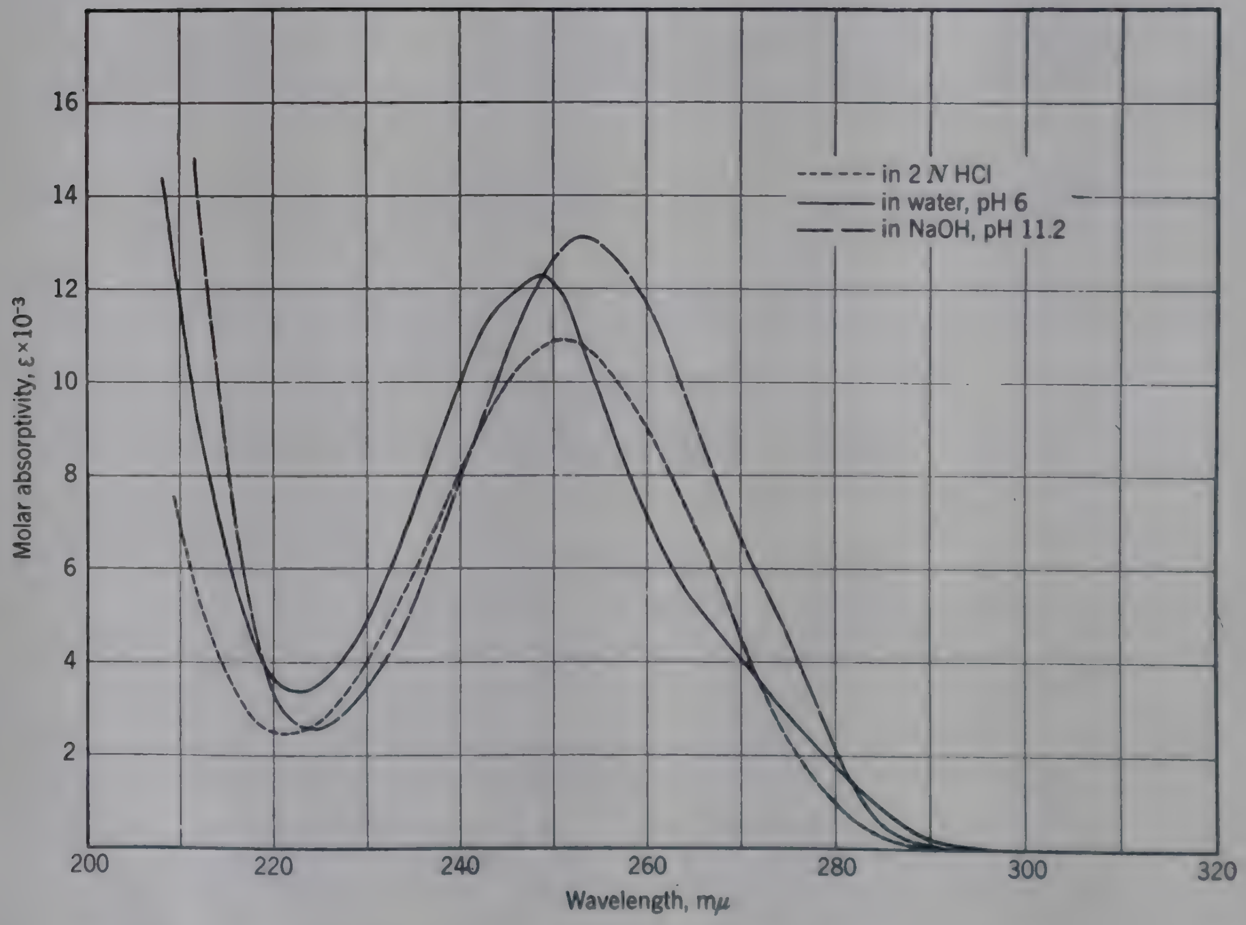

FIG. 12. Inosine (Johnson, unpublished).

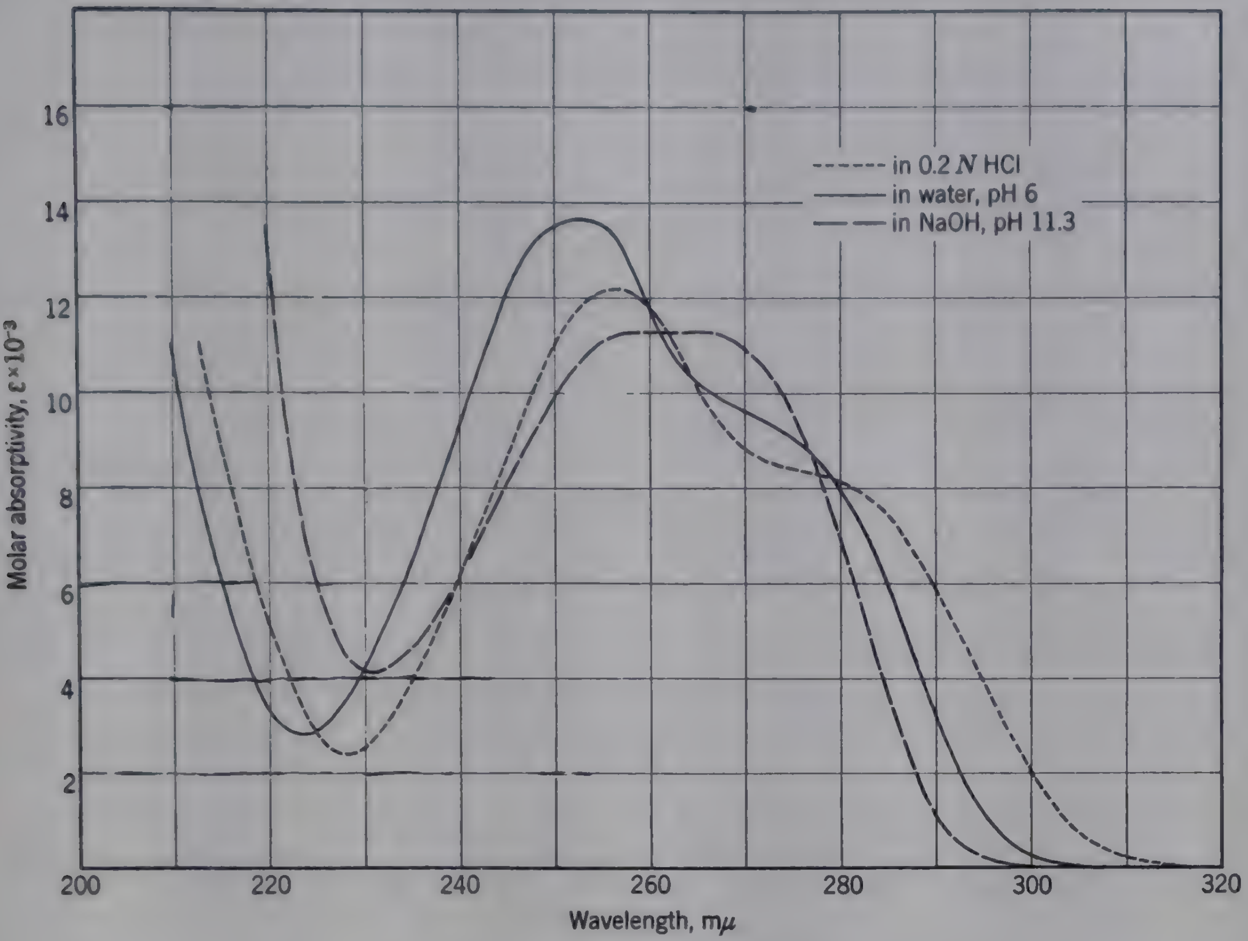

FIG. 13. Guanosine (Johnson, unpublished).

out by comparing their absorption spectra with those of the corresponding methyl-substituted compounds (Gulland *et al.*[37,41,45]).

It may perhaps be pointed out here that the deduction of Press and Butler,[46] on spectroscopic grounds, that the ring system of guanosine remains intact after treatment with di(2-chloroethyl)methylamine seems ill-founded. From the data given, there seems no reason to assume that the iminazole ring is unbroken, since pyrimidine derivatives frequently possess absorption maxima at longer wavelengths than purines.

3. NUCLEOTIDES

The separation of the phosphate group in a nucleotide from the effective ultraviolet chromophore by the saturated carbon chain of the sugar residue may be expected to ensure that its effect on the absorption characteristics will be small. This is in fact the case, and many nucleotides may be regarded as barely distinguishable from their parent nucleosides in the present state of ultraviolet spectroscopic technique.

The phosphate group may be substituted in the 2′-, 3′-, or 5′-position in ribonucleotides, and Markham and Smith[47] have recently shown that cyclic 2′,3′-monohydrogen phosphate esters may also be isolated from enzymic or

[45] J. M. Gulland and E. R. Holiday, *J. Chem. Soc.* **1936,** 765.

[46] E. M. Press and J. A. V. Butler, *J. Chem. Soc.* **1952,** 626.

[47] R. Markham and J. D. Smith, *Biochem. J.* **52,** 552 (1952).

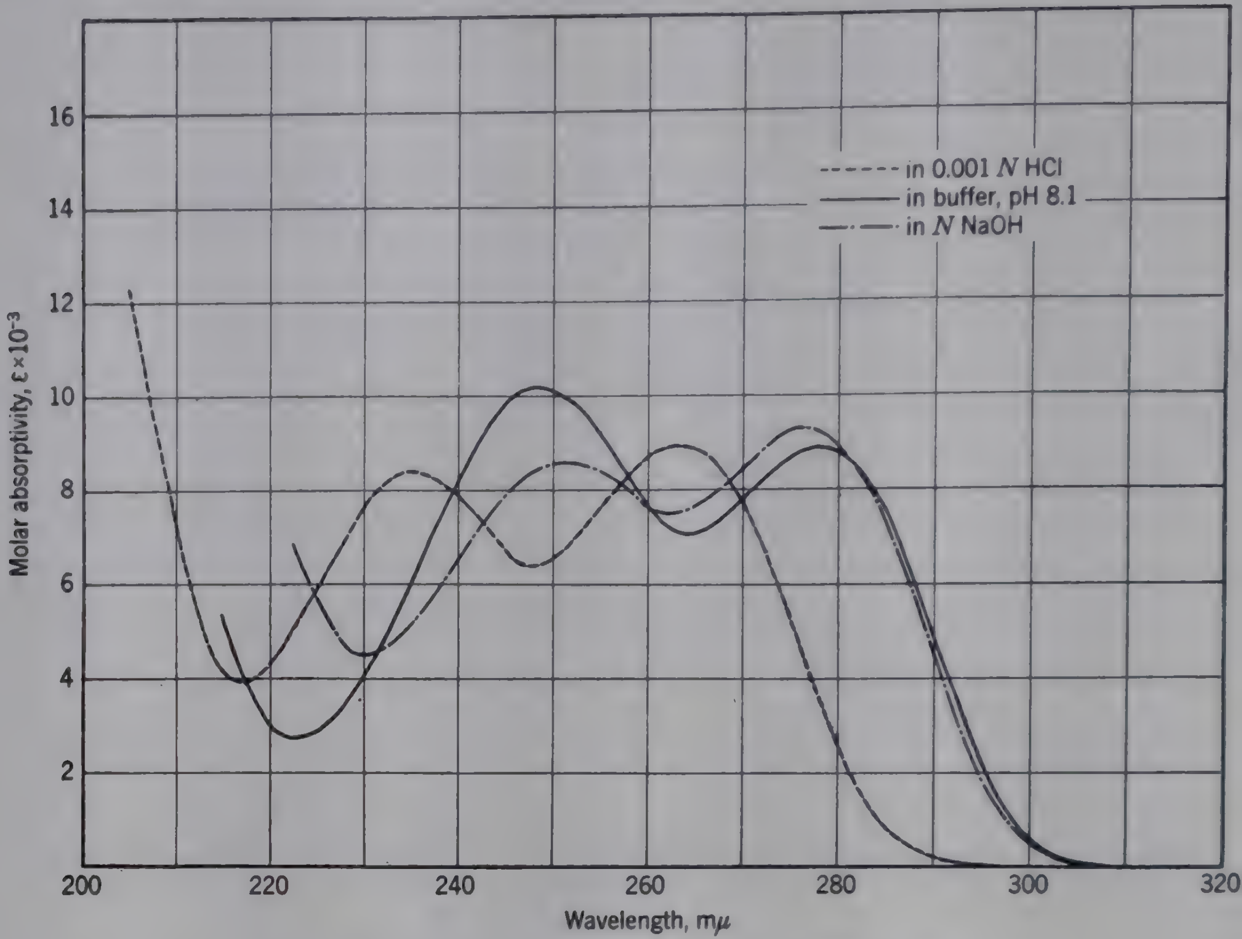

FIG. 14. Xanthosine (Johnson, unpublished).

alkaline digests of PNA. These have absorption spectra very similar t those of the other isomers. In addition to the hydroxyl and amino pK's c the bases and the sugar dissociation, further pK's are contributed in th nucleotides by the phosphate dissociations. These would again not be expected to influence the spectrum greatly, but inspection of the absorbanc ratios in Table III for the uridylic and thymidylic acids shows that change occur between pH 2 and pH 7 which are not attributable to any other cause

Separation techniques for the isomeric nucleotides have been worked ou only quite recently, and much of the spectroscopic work has therefore bee done on mixtures of unknown composition (Kerr *et al.*,[22] Ploeser and Loring,[28] Chargaff and co-workers,[48] Dunn and co-workers[49]). Kalckar[38] give data for adenylic acid-5′, inosinic acid-5′, and also ATP, and Kaplan *et al.* give a curve for ITP. Data for cytidylic acids *a*(2′) and *b*(3′) have bee published by Loring *et al.*,[43] who found that at pH 1 the 3′-isomer gav results very close to those of cytidine. Fig. 1 shows the curves for the isomeric cytidylic acids (Cohn, private communication), and a comparisor

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[48] B. Magasanik, E. Vischer, R. Doniger, D. Elson, and E. Chargaff, *J. Biol. Chem* **186,** 37 (1950).

[49] A. Deutsch, R. Zuckermann, and M. S. Dunn, *Anal. Chem.* **24,** 1769 (1952).

[50] N. O. Kaplan, S. P. Colowick, and M. M. Ciotti, *J. Biol. Chem.* **194,** 579 (1952).

Nucleotide	Absorbance ratios[a]									ϵ_{260}		
	A_{250}/A_{260}			A_{280}/A_{260}			A_{290}/A_{260}					
	pH 2	pH 7	pH 12	pH 2	pH 7	pH 12	pH 2	pH 7	pH 12	pH 2	pH 7	pH 12
Adenylic 2′	0.85	0.80	0.80	0.23	0.15	0.15	0.038	0.009	—	14.2	15.0	15.0
Adenylic 3′	0.85	0.80	0.80	0.22								
Adenylic 5′	0.84	0.79	0.79	0.22								
Cytidylic 2′	0.48	0.90	0.90	1.80	0.85	0.85	1.22	0.26	0.26	6.8	7.6	7.6
Cytidylic 3′	0.45	0.86	0.86	2.00	0.93	0.93	1.43	0.30	0.30			
Cytidylic 5′	0.46	0.84	0.84	2.10	0.99	0.99	1.55	0.33	0.33	6.2	7.4	—
Deoxycytidylic	0.43	0.82	0.82	2.12	0.99	0.99	1.55	0.30	0.30	—	—	—
Cytidine di P	0.46	0.88	—	2.00	0.93	—	1.48	0.32	—	—	—	—
Uridylic 2′	0.80	0.78	0.85	0.28	0.30	0.25	0.03	0.03	0.02	10.0	10.0	7.4
Uridylic 3′	0.76	0.73	0.83	0.32	0.35	0.28						
Uridylic 5′	0.74	0.73	0.82	0.38	0.40	0.33						
Uridine di P	0.80	—	—	0.34	—	—	0.04	—	—	—	—	—
Thymidylic	0.64	0.65	0.74	0.72	0.73	0.67	0.23	0.24	0.17	8.4	8.4	6.7
Thymidine di P	0.65	—	—	0.70	—	—	0.20	—	—	—	—	—
5-Methylcytidylic	0.36	0.96	0.96	3.15	1.52	1.52	3.4	1.02	1.02	—	—	—
	pH 1	pH 7	pH 12	pH 1	pH 7	pH 12	pH 1	pH 7	pH 12	pH 1	pH 7	pH 12
Guanylic 2′	0.90	1.15	0.89	0.68	0.68	0.60	0.48	0.285	0.11	11.8[b]	11.4[b]	11.2[b]
Guanylic 3′	0.93			0.69			0.49					
Guanylic 5′	0.99			0.70			0.51					

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[a] Wavelengths in mμ.

[b] Johnson (unpublished). Sample probably principally 3′-isomer.

of Table II with Table III, also compiled from data kindly provided b Cohn, shows that in general the nucleotide in which the phosphate is nea est the base, i.e., the 2′, shows the greatest differences from the correspon ing nucleoside, which might perhaps be expected. The curves for guanyli acid (Fig. 19) were determined in this laboratory. Although separation c the isomers was not specifically attempted, the compound was purified b ion-exchange chromatography and appears from the absorbance ratios t consist very largely of the 3′-isomer. The very close resemblance to guano sine (Fig. 13) may be noted.

The absorption curves given for 5-methylcytidylic acid (Fig. 20, fron Cohn[31]) appear to be the only ones so far available for a deoxynucleotide These are however relative, although a very close estimate of absolut values may be obtained from the corresponding nucleoside.

III. Nucleic Acids and Polynucleotides

1. General

As is to be expected from their content of purines and pyrimidines, intac nucleic acids show selective absorption in the λ 260-mμ region.

Spectrophotometry has been widely used for estimating the purine an pyrimidine bases obtained from hydrolytic degradation of nucleic acids [Cf. Chapters 5–7, 10, and 11.] Reviewing in 1940 the small amount of prio spectrophotometric work, Loofbourow[51] concluded that the absorptivity c a nucleic acid was, to a close approximation, the sum of the contribution of the constituent bases, assuming the tetranucleotide theory of their com position to be correct. The spectrophotometric analysis of an intact nuclei acid would thus constitute a difficult four- or perhaps five-component analy sis. This does not prove possible, however, since nucleic acids, both DNA[51 and PNA, show lower absorptivities, by up to 35%, than the value cal culated from summing the molar absorptivities of the contained nucleotide (as determined by separation and analysis). Possible causes for this anomal are of interest in relation to theories of the structure of nucleic acids. Th reduction in total absorptivity may be regarded as arising from modifi cations of the chromophoric groups of one or more of the individual nucleo tide units in the macromolecule. Further, it appears that the absorptivit value of a nucleic acid is very sensitive to its previous treatment and i some respects may be more easily affected than other physical propertie Although the results of investigations of these phenomena are beginning t appear, it should be emphasized that complete explanation of the anomalou

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[51] J. R. Loofbourow, *Revs. Mod. Phys.* **12**, 320 (1940).

[51a] The abbreviation DNA may be understood to refer to the sodium salt where ap propriate.

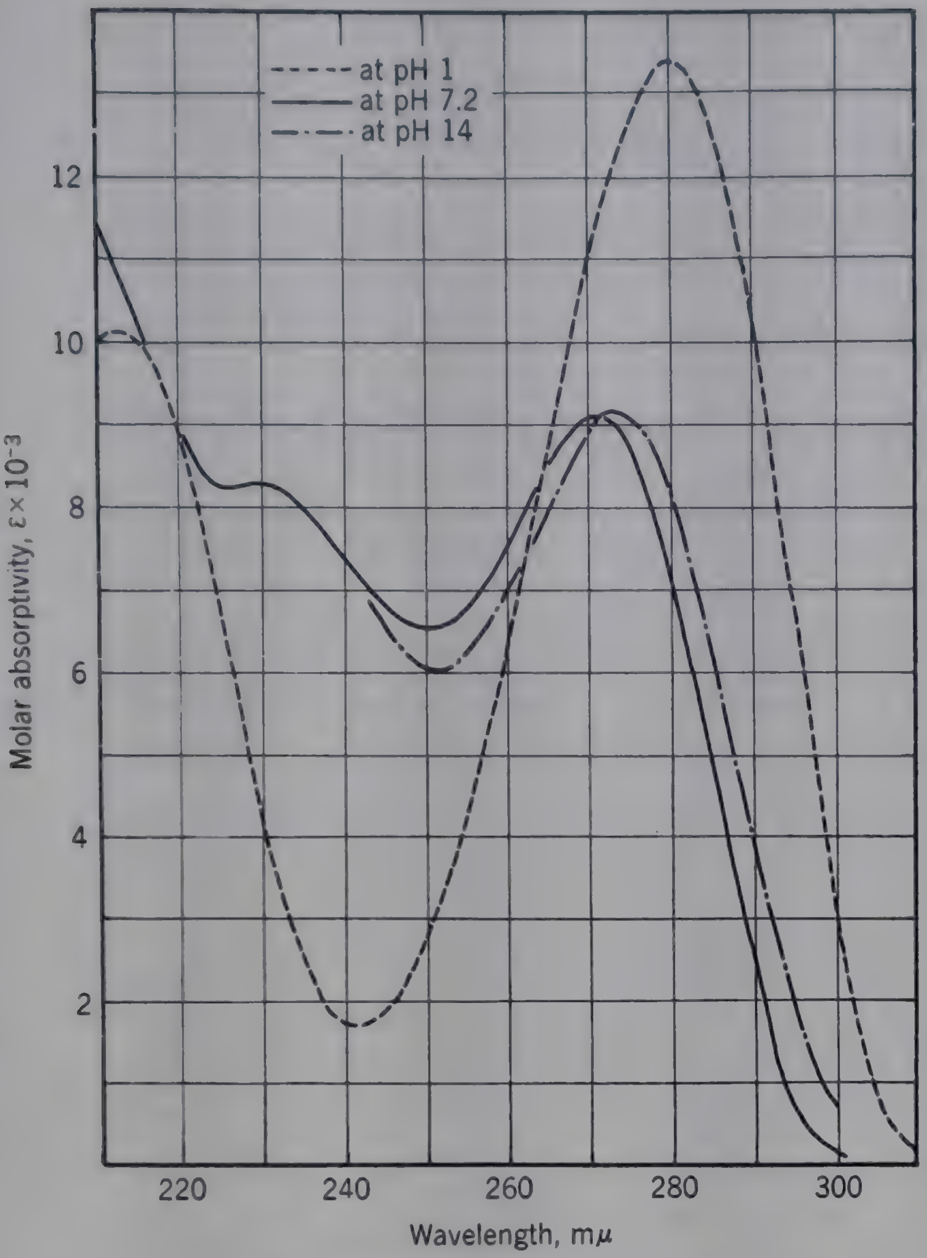

FIG. 15. Cytidine (Fox and Shugar[16]).

ultraviolet absorption characteristics of nucleic acids is not possible at the moment. The recent spectrophotometric studies by Cavalieri,[52] Shack *et al.*,[53,54] Thomas,[55] and Lawley (working in Jordan's laboratory),[56] indicate that hitherto unsuspected factors may cause irreversible changes in the absorption spectrum of DNA. These changes in the macromolecular structure we may term, from protein analogies, denaturation. Consequently, many preparations and physical studies of DNA have been carried out under conditions which have since been found to cause denaturation. In the light of these findings the ultraviolet absorption characteristics of a

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[52] L. F. Cavalieri, *J. Am. Chem. Soc.* **74,** 1244 (1952).

[53] J. Shack, R. J. Jenkins, and J. M. Thompsett, *J. Biol. Chem.* **198,** 85 (1952).

[54] J. Shack, R. J. Jenkins, and J. M. Thompsett, *J. Biol. Chem.* **203,** 373 (1953).

[55] R. Thomas, *Bull. soc. chim. biol.* **35,** 609 (1953).

[56] P. D. Lawley, Ph.D. Thesis, Nottingham University, 1953.

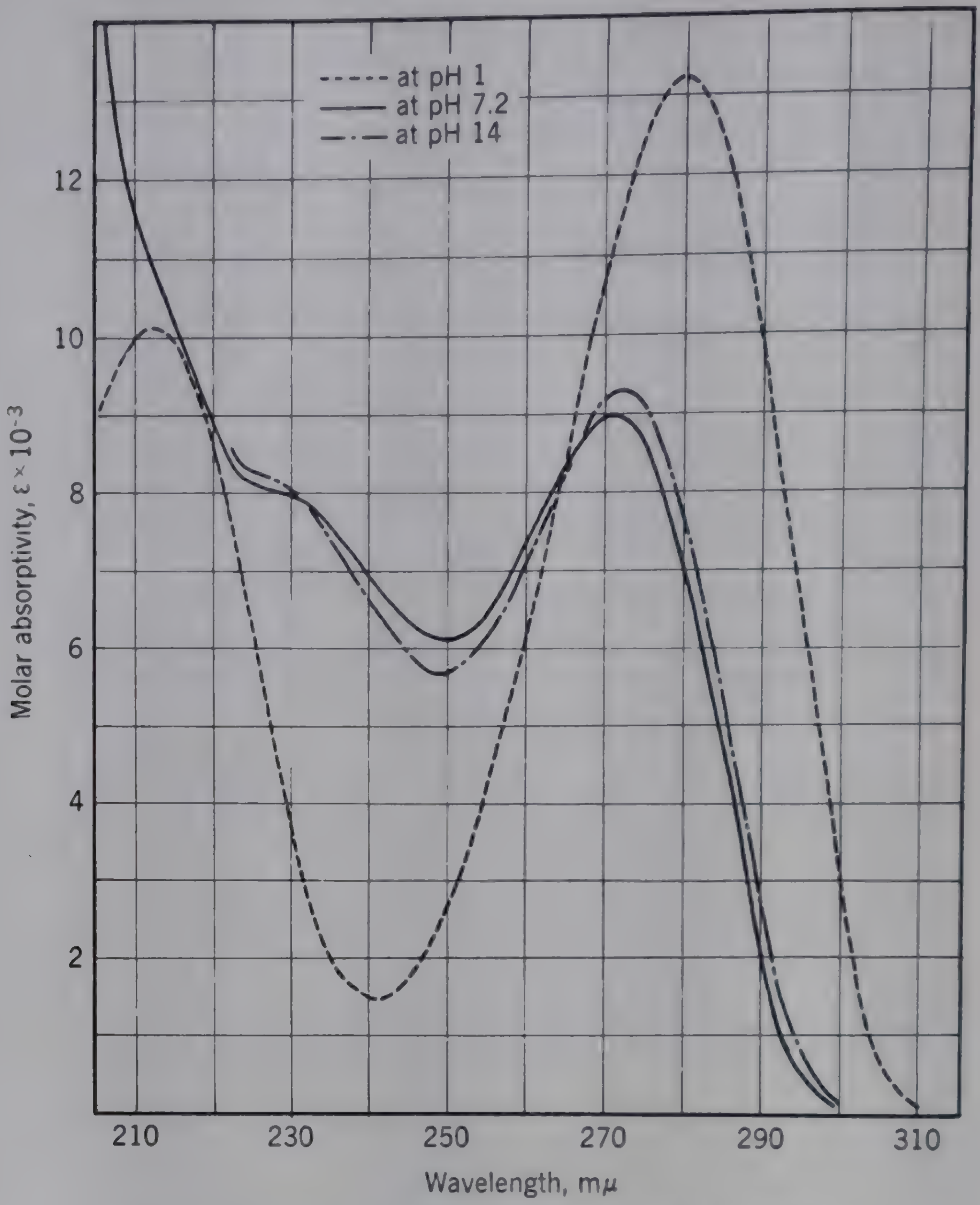

Fig. 16. Cytosine deoxyriboside (Fox and Shugar[16]).

nucleic acid acquire an importance comparable with the viscosity and the sedimentation constant in the assessment of the macromolecular status of the nucleic acid.

2. The Absorption Spectra of Nucleic Acids

Because of their varying content of water the molar absorptivities of nucleic acids cannot reliably be based on weight. A more practicable convention has been suggested by Chargaff and Zamenhof.[57] Since nucleic acids contain one phosphorus atom per nitrogenous base, these workers relate the absorbance of a solution of nucleic acid to phosphorus concentration and derive a molar absorptivity, $\epsilon(P)$, for nucleic acid, based on one gram-atom of phosphorus per liter.

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[57] E. Chargaff and S. Zamenhof, *J. Biol. Chem.* **173**, 327 (1948).

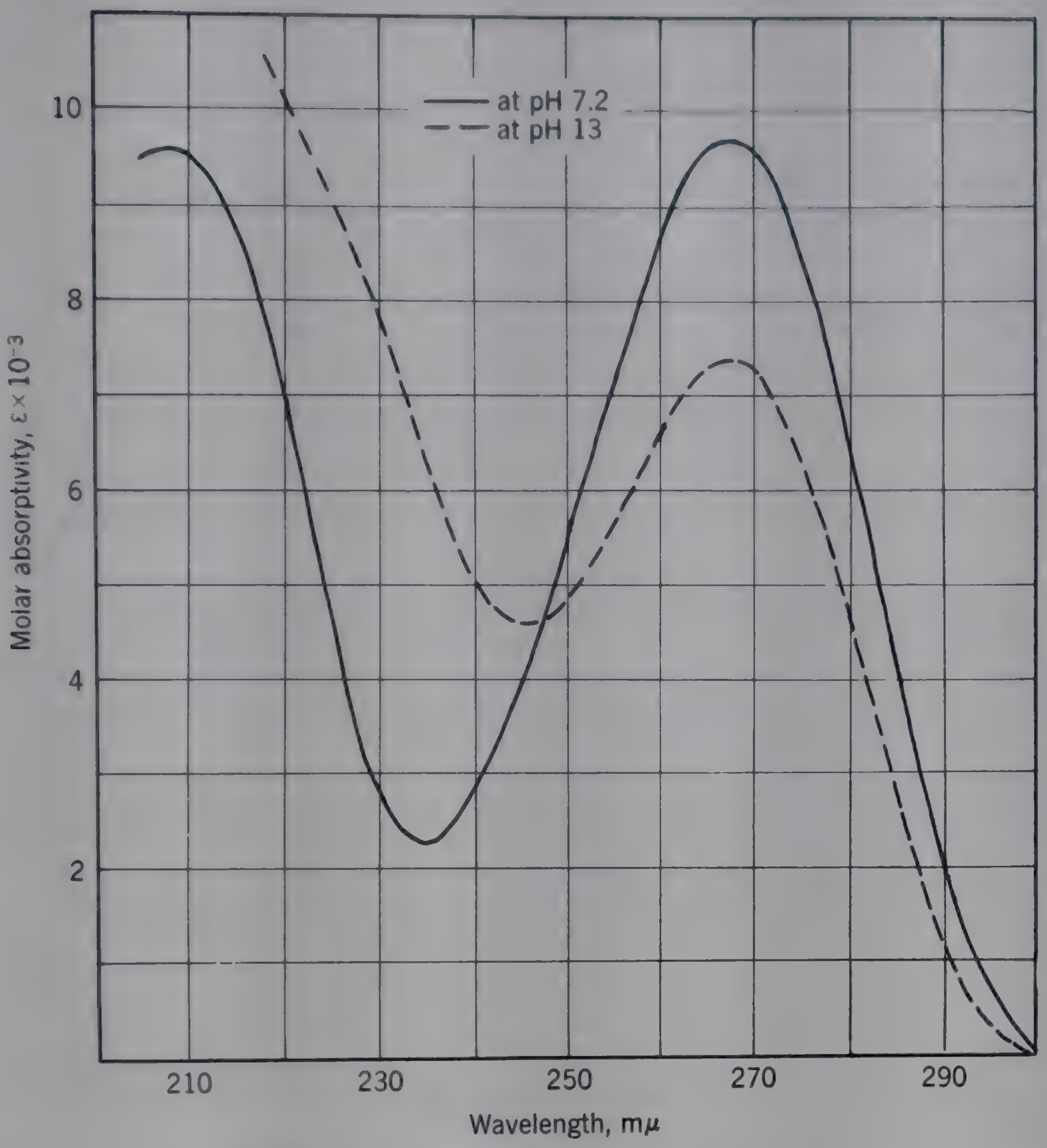

FIG. 17. Thymidine (Fox and Shugar[16]).

As defined by Chargaff and Zamenhof

$$\epsilon(\mathrm{P}) = A/C \cdot d$$

A and d have been defined above; C is measured in gram-atoms of phosphorus per liter.

A review of published values of ϵ(P) shows very wide variation, from 6,000 to 8,000 for DNA, and from 7,000 to 10,000 for PNA. When results have not been expressed in accordance with this convention, comparison with other work is often impossible. The ϵ(P) value of a nucleic acid measured under standard conditions may eventually serve as an index of the degree of denaturation.

Two general conclusions may be stated:

(a) All recorded values of ϵ(P) for intact DNA and PNA are lower than

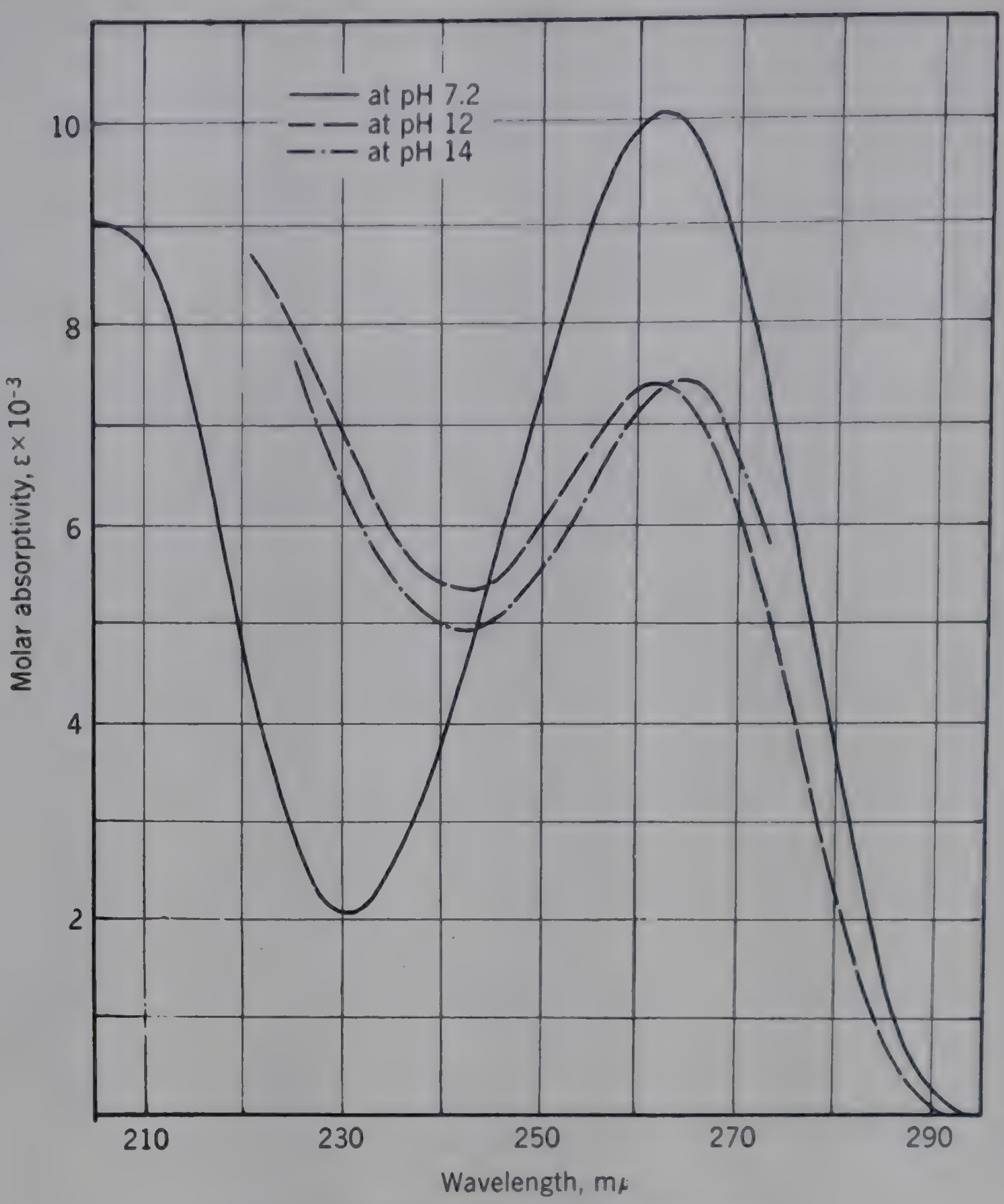

FIG. 18. Uridine (Fox and Shugar[16]).

the values calculated from the summation of the absorptivities of the con tained purine and pyrimidine nucleotides.

(b) Values of $\epsilon(P)$ show poor reproducibility even from the same prepara tion of nucleic acid.[54,57,58] It is of interest therefore to find out at what leve of hydrolytic degradation the anomalously low $\epsilon(P)$ of nucleic acid is abol ished.

Kunitz[59] had observed slight increases in the absorption maxima o yeast PNA samples when depolymerized with ribonuclease, although her the primary effect was a shift of the entire curve to shorter wavelengths Oster and Grimsson[60] and Ogur and Rosen[61] found that other PNA sam

[58] A. M. Marko and G. C. Butler, *J. Biol. Chem.* **190,** 165 (1951).

[59] M. Kunitz, *J. Biol. Chem.* **164,** 563 (1946).

[60] G. Oster and H. Grimsson, *Arch. Biochem.* **24,** 119 (1949).

[61] M. Ogur and G. Rosen, *Arch. Biochem.* **25,** 262 (1950).

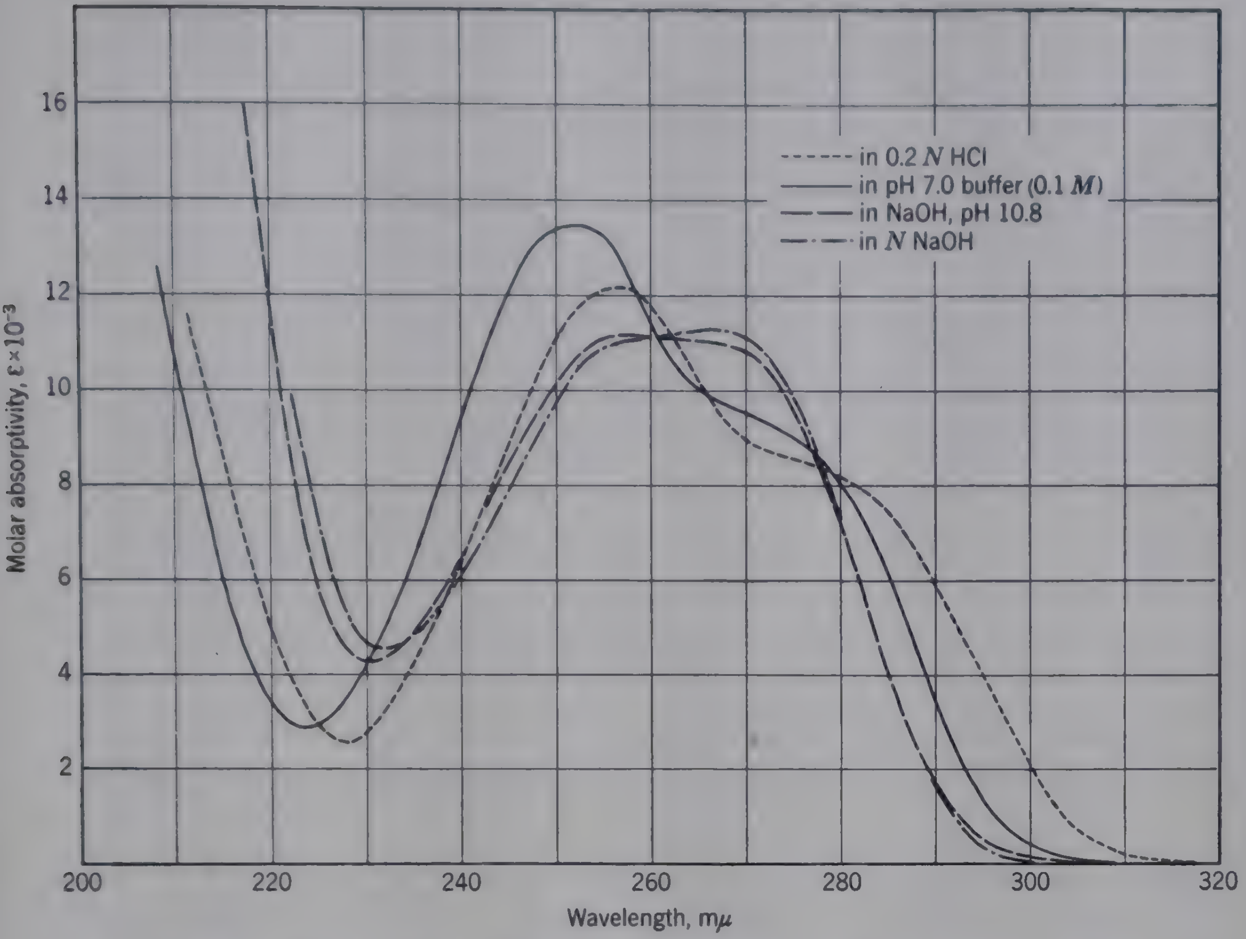

FIG. 19. Guanylic acid (largely 3′-isomer) (Johnson, unpublished).

ples showed similar increases in absorption of from 5 to 15% on enzymic degradation at neutral pH or on partial alkaline or acid hydrolysis. Kunitz[62] also first observed that depolymerization of DNA at pH 5 by deoxyribonuclease was accompanied by an ultimate increase in absorption at 260 mμ of nearly 30%, and increases of a similar order were reported for both DNA and PNA by Tsuboi.[63]

The relation between the absorptivity of the intact nucleic acids and the value obtained by summing the absorptivities of the constituent nucleotides was first pointed out, for a number of PNA samples, by Magasanik and Chargaff.[64] They found that the absorbance of the total alkaline hydrolysis products was invariably greater by some 24–37% than that of the original sample, and that if, after partial enzymic hydrolysis of the PNA, the products were separated into dialyzable and nondialyzable fractions, the increase in absorbance or "hyperchromic effect" on total alkaline hydrolysis was almost negligible for the former and very marked for the latter. These latter polynucleotide "cores" were especially rich in guanylic acid, and it appeared that this hyperchromic effect might be especially associated with

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[62] M. Kunitz, *J. Gen. Physiol.* **33,** 349 (1950).

[63] K. K. Tsuboi, *Biochim. et Biophys. Acta* **6,** 202 (1950).

[64] B. Magasanik and E. Chargaff, *Biochim. et Biophys. Acta* **7,** 396 (1951).

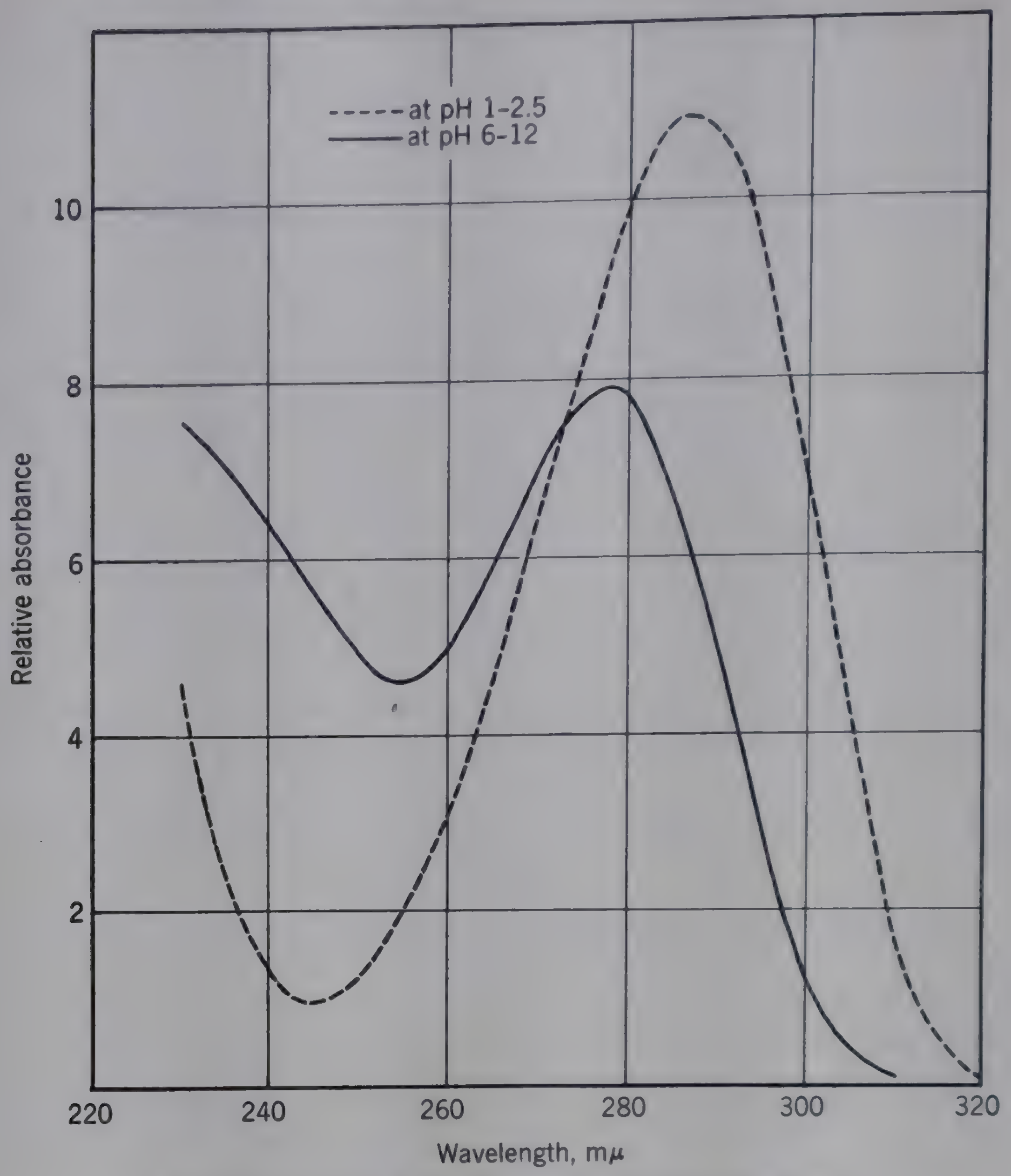

FIG. 20. Deoxy-5-methylcytidylic acid (Cohn[31]).

the presence of this nucleotide, since the nondialyzable PNA cores obtaine by partial alkaline hydrolysis, which were rich rather in adenylic than guanylic acid, showed a much smaller hyperchromic effect than either th parent PNA or the enzymic core.

In the case of still smaller fragments there seems to be no evidence of anomalou absorption. Merrifield and Woolley[65] have confirmed that a number of dinucleotid and a trinucleotide derived from DNA show absorptivities which are within exper mental error the sums of those of their consistent mononucleotides (see also Elmo and Todd[66]), and Sinsheimer and Koerner[67] make the same claim for di-deoxyrib

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[65] R. B. Merrifield and D. W. Woolley, *J. Biol. Chem.* **197**, 521 (1952).

[66] D. T. Elmore and A. R. Todd, *J. Chem. Soc.* **1952**, 3681.

[67] R. L. Sinsheimer and J. F. Koerner, *J. Am. Chem. Soc.* **74**, 283 (1952).

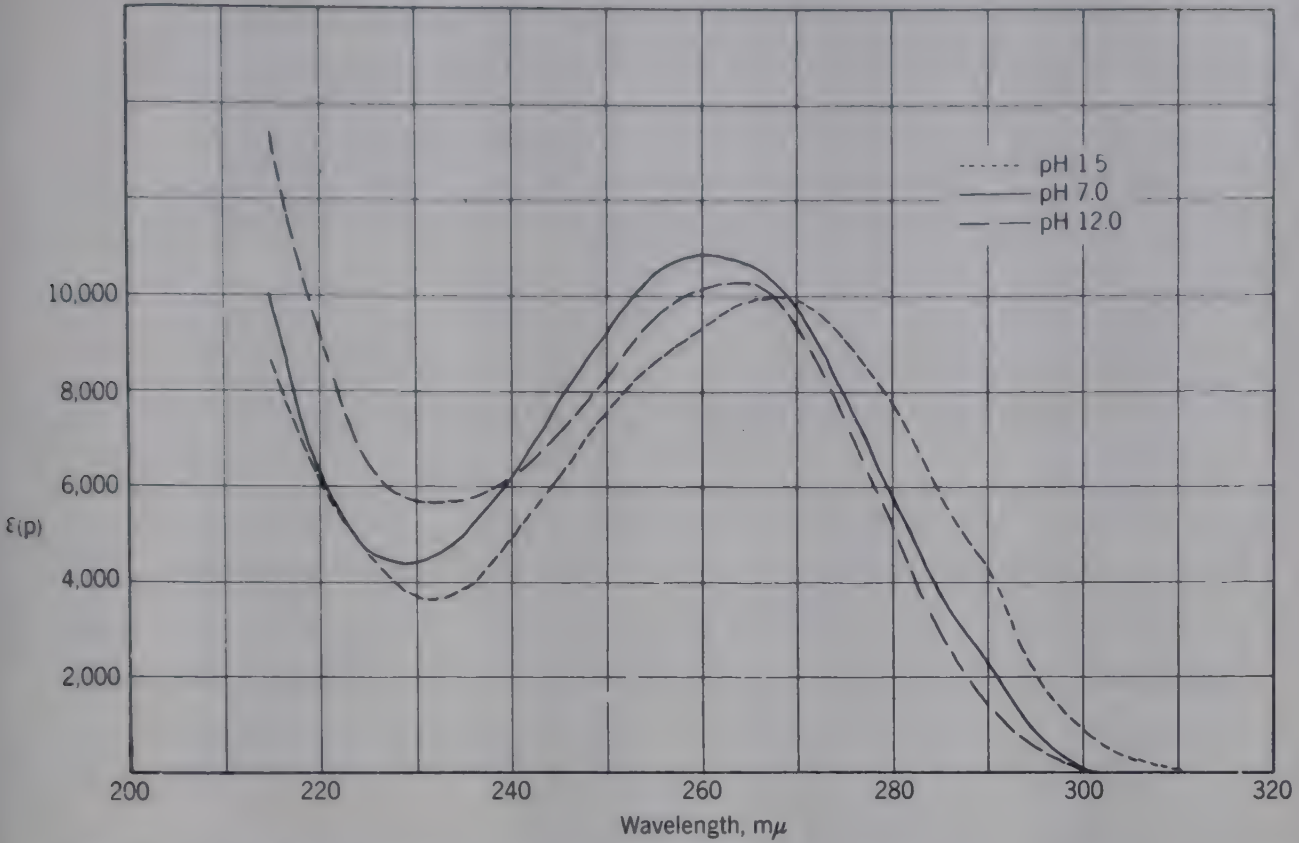

FIG. 21. Calculated absorption curves for a mixture of nucleosides containing adenosine, guanosine, cytidine, and thymidine in the molar proportions 4:3:3:4. At pH 1.5 values for adenine and guanine are employed.

nucleotides. More recently Smith and Allen[68] state that the absorptivities of polyribonucleotides containing up to 20 nucleotide residues appear to be normal, and the same may be concluded of the "apurinic acid" of Chargaff and co-workers,[69] which, however, can scarcely be regarded as a normal polynucleotide.

This absorbance anomaly therefore appears to be essentially a feature of polynucleotides of relatively high molecular weight and probably denotes some considerable degree of intramolecular organization. It is not due to the binding of the nucleotides by glycosido-phosphate ester linkages. The causes for it may therefore be of direct concern when considering theories of the structure of nucleic acids.

The work reviewed below has been mainly concerned with calf thymus DNA, which is perhaps the most readily accessible highly polymeric nucleic acid. In general, the ribonucleic acids are far less well-characterized materials, as a class, than the deoxyribonucleic acids. For this reason, although the causes of the absorptivity anomalies may be similar in both types of nucleic acid, the results of studies of the effects of pH, cations, heat, and

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[68] K. C. Smith and F. W. Allen, *J. Am. Chem. Soc.* **75,** 2131 (1953).

[69] C. Tamm, M. E. Hodes, and E. Chargaff, *J. Biol. Chem.* **195,** 49 (1952).

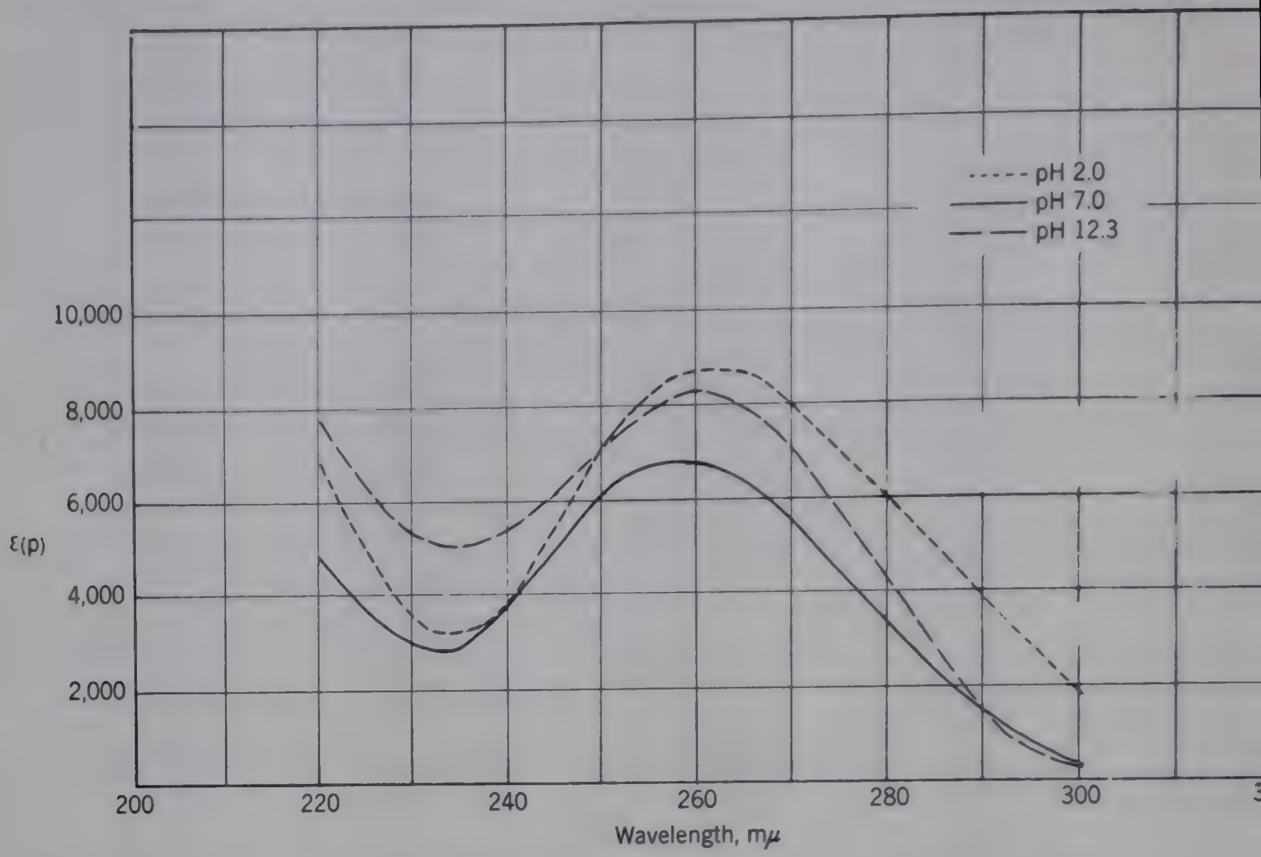

FIG. 22. Calf thymus DNA (Shack and Thompsett[70]).

dehydration on the ε(P) of PNA will be difficult to interpret until an u
denatured form (Cf. Kay and Dounce[71]) can be unequivocally recognize

3. EFFECT OF pH ON THE ε(P) OF DNA

In Fig. 21 the absorption curve of a mixture of nucleotides correspondin
in nitrogen base content to a typical DNA is shown at three pH value
In Fig. 22 is shown the absorption curve of a calf thymus DNA preparatio
under similar conditions. These curves clearly indicate that the marke
changes in absorptivity of DNA with change of pH cannot be attribute
simply to ionization of the purine and pyrimidine bases. The effects
change in pH when summed for the four principal nucleotides in a nucle
acid (Fig. 21) result in a small change in absorptivity and a slight shift
wavelength, whereas there are gross changes for DNA itself under simila
conditions. Further it can be seen that on exposing DNA to acid or alka
its absorption curve approaches more closely to the calculated one. It
noteworthy, however, to find that at least part of the well-known anomalou
electrometric titration curve of DNA[72,73] may also be obtained spectr

[70] J. Shack and J. M. Thompsett, *J. Biol. Chem.* **197**, 17 (1952).
[71] E. R. M. Kay and A. L. Dounce, *J. Am. Chem. Soc.* **75**, 4041 (1953).
[72] J. M. Gulland, D. O. Jordan, and H. F. W. Taylor, *J. Chem. Soc.* **1947,** 1131.
[73] W. A. Lee and A. R. Peacocke, *J. Chem. Soc.* **1951,** 3361.

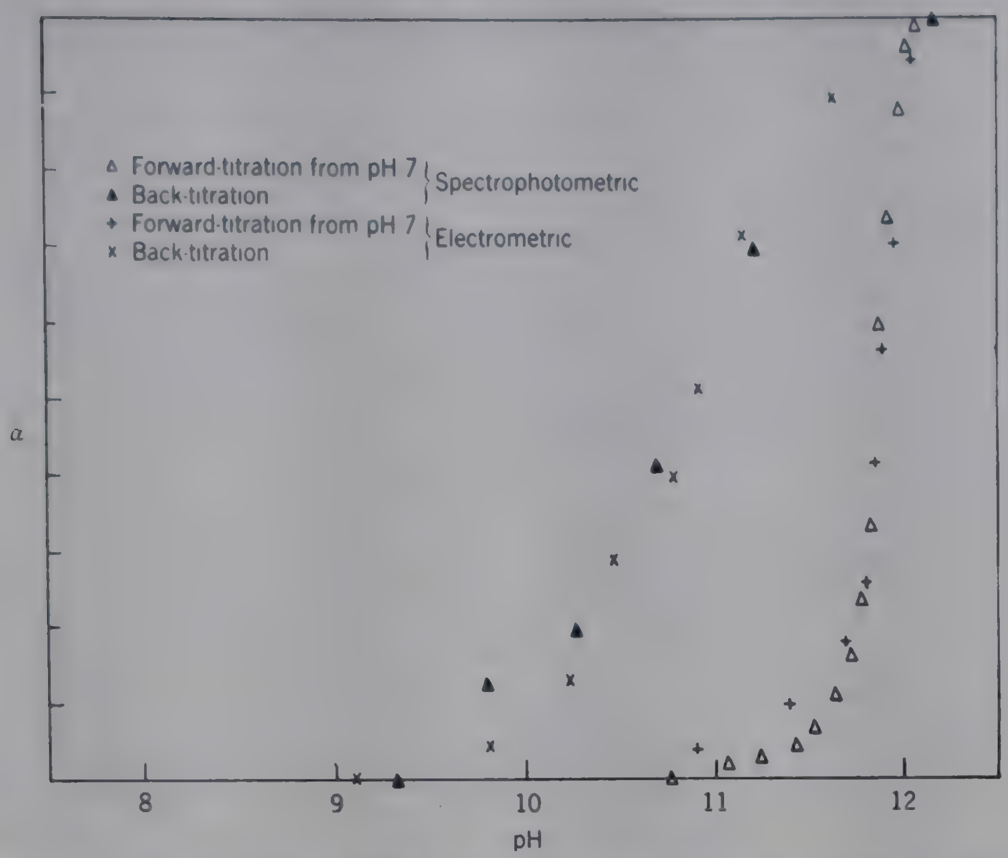

FIG. 23. Spectrophotometric and electrometric titration data for calf thymus DNA (Shack and Thompsett[70]). (α represents the proportion of the high-ϵ(P) form of the nucleate, or the proportion of nucleate anion.)

photometrically.[53,54] Fig. 23, taken from a paper by Shack and Thompsett,[70] compares the electrometric and spectrophotometric titration curves of DNA when titrated with alkali. It can be seen from these results that over the pH range 9–12 the increase in ϵ(P) corresponds with ionization, presumably of the hydroxyl groups of guanosine (pK_a 9.5) and thymidine (pK_a 9.8). It is evident that the pK_a values of these groups are significantly higher in the DNA macromolecule.

On the acid side of neutrality comparative electrometric and spectrophotometric titration data at pH values lower than 3.5 are lacking and such a correspondence does not emerge.

Fig. 24 (excluding curve 1) shows spectrophotometric titration curves obtained in different laboratories [56,74,75] on samples of calf thymus DNA in absence of salts. These show that on titration of DNA with acid at the lowest possible salt concentration, the ϵ(P) of the nucleic acid begins to rise at a pH as high as 8.0 and has almost attained its maximum value at pH 5.0. A typical electrometric titration [70,72,73] on the other hand shows that in this pH region no ionic groups are being titrated. On the acid side of pH 8.0, therefore, it appears that an increase in ϵ(P) occurs, in the absence of salts, at a higher pH than ionization. The effect of the addition of salts

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[74] G. Frick, *Biochim. et Biophys. Acta* **8,** 625 (1952).

[75] E. R. Blout and A. Asadourian, *Biochim. et Biophys. Acta* **13,** 161 (1954).

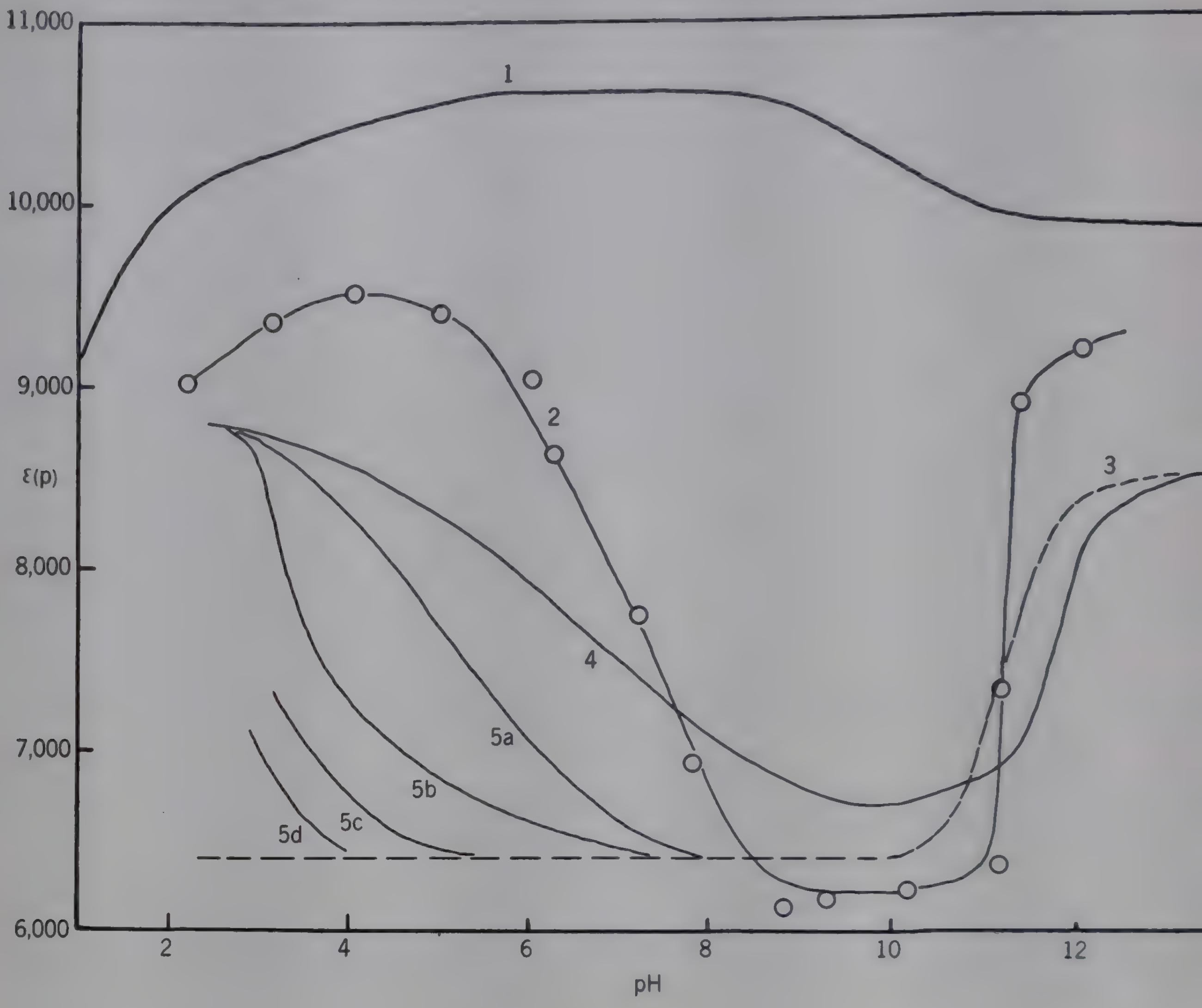

Fig. 24. Variation of ε(P) of calf thymus DNA with pH.

(1) Calculated for a mixture of nucleosides containing adenosine, guanosine, cyt
dine, and thymidine in the molar proportions 4:3:3.4. Below pH 2 values for adeni
and guanine are employed.

(2) Titration of calf thymus DNA in absence of salt (Blout and Asadourian[75]).

(3) Titration in 1 M NaCl solution (Frick[74]).

(4) Titration in absence of added salts.

(5a) in 10^{-5} M $\mathrm{MgSO_4}$.

(5b) In 3.4×10^{-3} M $\mathrm{MgSO_4}$.

(5c) In 0.1 M NaCl.

(5d) In M NaCl (Lawley[56]).

is to decrease the pH value at which the increase in ε(P) starts to occu
(see below). These observations indicate that there are two independen
processes, ionization on the one hand and a change of ε(P) on the othe
On the alkaline side the process responsible for the changes in ε(P) an
ionization is concurrent with change in pH, whereas on the acid side,
any rate in the absence of salts, they are independent.

The lowered absorptivity of the bases, resulting in the low $\epsilon(P)$ of DNA is most plausibly interpreted as due to a disturbance of the tautomeric equilibrium of one or more of the bases. It is difficult to envisage any other type of explanation: no exception to Lambert's law has been recorded so that the lowering of $\epsilon(P)$ cannot be accounted for on the basis of "optical" interference between chromophoric groups (i.e., screening affects[56]).

Purine and pyrimidine bases exhibit keto–enol tautomerism, and it has been shown[7,9] that free pyrimidines occur in a predominantly keto form, although ionization to the anion may be presumed to involve an increase in enolic character. [Cf. *Jordan*, Chapter 13.] The observation that the electrometric and spectrophotometric titrations proceed concurrently in the alkaline pH region suggests that hydrogen ions are being released from the ionizable hydroxyl groups (with modified pK_a values) of bases in a predominantly enolic form. Such a degree of enolic character for the bases in intact DNA would correspond, at least qualititively, to certain structures for DNA proposed by Stern[76] and by Watson and Crick.[77] In the structure proposed by the latter, specific pairs of adjacent bases are hydrogen-bonded through the 1,1- and 6,6-positions. [Compare Fig. 8 in Chapter 13.] The effect of ionization is always to raise the value of $\epsilon(P)$, but the salt effects at constant pH indicate that the factors operating in the native macromolecule which are responsible for the absorption anomaly can also be counteracted (in part reversibly) in other ways.

4. Effect of Salts on the Absorptivity of DNA

It has been known for some time that exposure of nucleic acids to extremes of pH, temperature, dehydration, etc. causes marked irreversible changes in physical properties, including $\epsilon(P)$. More recently the effects of salts in particular on the $\epsilon(P)$ value of DNA have been studied in several laboratories.

Thomas[55,78] has shown that exposure of DNA to low sodium chloride concentration ($<10^{-3}$ M) results in irreversible changes in its structure. The evidence for this may be summarized as follows. A DNA preparation, which has never been exposed to low salt concentration, extremes of pH, or other denaturing influences, when dissolved in sodium chloride solution (concentration $>10^{-3}$ M) has an $\epsilon(P)$ value in the region of 6000. Such a solution is quite stable for several days as judged by the constancy of $\epsilon(P)$ and by the fact that increasing the concentration of sodium chloride does not lower the $\epsilon(P)$ value. However, a solution of DNA in which the concentration of sodium chloride is less than 10^{-3} M has an $\epsilon(P)$ value considerably higher than 6000, reaching a value of 8000 at a concentration of *ca.* 10^{-5} M. When

[76] K. G. Stern, *Yale J. Biol. Med.* **19,** 937 (1947).

[77] J. D. Watson and F. H. C. Crick, *Nature* **171,** 737, 964 (1953).

[78] R. Thomas, *Biochim. et Biophys. Acta* **14,** 231 (1954).

the concentration of sodium chloride in such a solution is subsequen raised, the ϵ(P) value is reduced, but not to the value that would have be found had the DNA not been previously exposed to the lower concentrati It may be concluded that the irreversible increase in ϵ(P) which occurs low salt concentration must be due to irreversible structural changes in t DNA molecule. The possibility that such a change is due to contaminati by depolymerizing enzyme has been suggested by Gilbert *et al.*[79] to expla the spontaneous changes in the viscosity of DNA dissolved in distilled wat

In the light of these results some doubt exists about the interpretati that can be placed on the careful work of Shack *et al.*[54] on the effects varying metal-ion concentrations on the absorptivity of DNA. Since th solutions were initially made up in distilled water, the DNA may ha been irreversibly changed to an unknown extent, though evidently n sufficiently to obscure the effects under study. Fig. 25 contains informati derived from their paper,[54] showing the lowering of ϵ(P) of DNA, initia dissolved in distilled water, following the addition of monovalent catio It also demonstrates the much greater activity in this respect of divale cations. In this connection Lawley[56] has shown that cations compete f the DNA anion in the order H^+, Mg^{++}, Na^+, K^+. Cavalieri[52] has shown th adding a large excess of magnesium sulfate to a solution of sodium DN lowers the pH. Fig. 24 shows that in the presence of metal cations the p value at which the increase in ϵ(P) commences is lowered. This figure al includes Frick's data[74] in molar sodium chloride solution. On the alkali side of neutrality metal cations have very little effect. In Fig. 26 are show titration curves illustrating the combined effects of variations in salt co centration and pH on the ϵ(P) value of DNA. The effect of exposure to lo salt concentration is to increase the ϵ(P) value at pH 6, although the fin value at low pH is independent of this prior treatment. Denaturation this manner thus reduces the eventual increase in ϵ(P) on acidificatio This behavior suggests itself as a useful test for the state of denaturati of a given sample of DNA. It is dissolved in 10^{-2} M sodium chloride and i absorbance determined at pH 3.0 and pH 7.0. The ratio (R_A) of these tw values may be taken as a measure of the extent of denaturation of th sample. From Fig. 26, R_A is seen to be 1.30 for Thomas' undenature material and 1.12 for the material used to obtain the other two curve Both Frick[74] and Shack *et al.*[54] have suggested that the ϵ(P) value of DNA preparation is a good indication of its state of denaturation. The te given here has the added advantage that phosphorus determinations a unnecessary. It is evident from the results of this work that metal catio play an important role in the stabilization of the structure of native DN

[79] L. Gilbert, W. G. Overend, and M. Webb, *Exptl. Cell Research* **2**, 349 (1951).

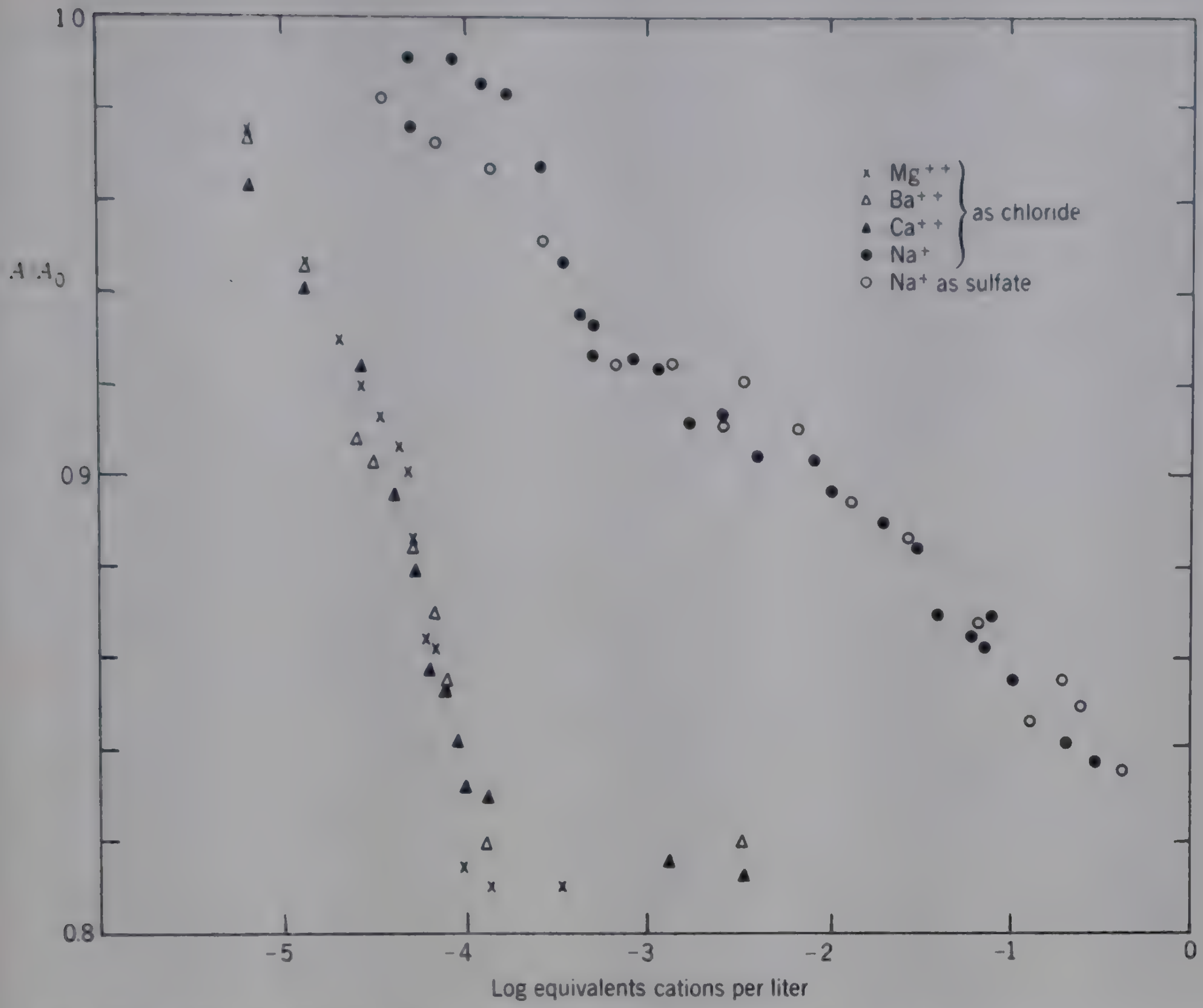

Fig. 25. Effect of metal cations on ϵ(P) of calf thymus DNA. The ordinate scale is the ratio of A, the absorbance in the presence of a given concentration of salt, to A_0, the absorbance in the absence of salt (Shack, Jenkins, and Thompsett[54]).

The nature of this role is still far from clear, but it is at least certain that the "ionic" history of a DNA preparation is of great importance.

The fact that divalent cations such as Ca^{++} and Mg^{++} stabilize DNA at much lower concentrations than do monovalent ions introduces an additional practical complication. Many preparations of DNA may have been dialyzed against tap water containing sufficient Ca^{++} ions to ensure stabilization. It may be expected that such preparations will be more stable at low salt concentrations than DNA that has not been so treated, and that, on subsequent dialysis against distilled water, they will tend to lose any remaining Na^+ ions before Ca^{++} ions.

Certain observations which were difficult to explain are now readily accounted for on the basis of the above findings. For instance it has been reported that the ϵ(P) of a preparation of DNA varies with the concentration, i.e., that Beer's law is not obeyed, but that in the presence of salt ϵ(P) is virtually constant over a wide range of concentration.[56,75,80]

It has also been observed that the ϵ(P) values of a given sample of DNA in distilled water solution are not reproducible.[54,58] Thomas[78] has shown that the extent of

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[80] J. Pouyet, G. Scheibling, and H. Schwander, *J. chim. phys.* **47**, 417 (1950).

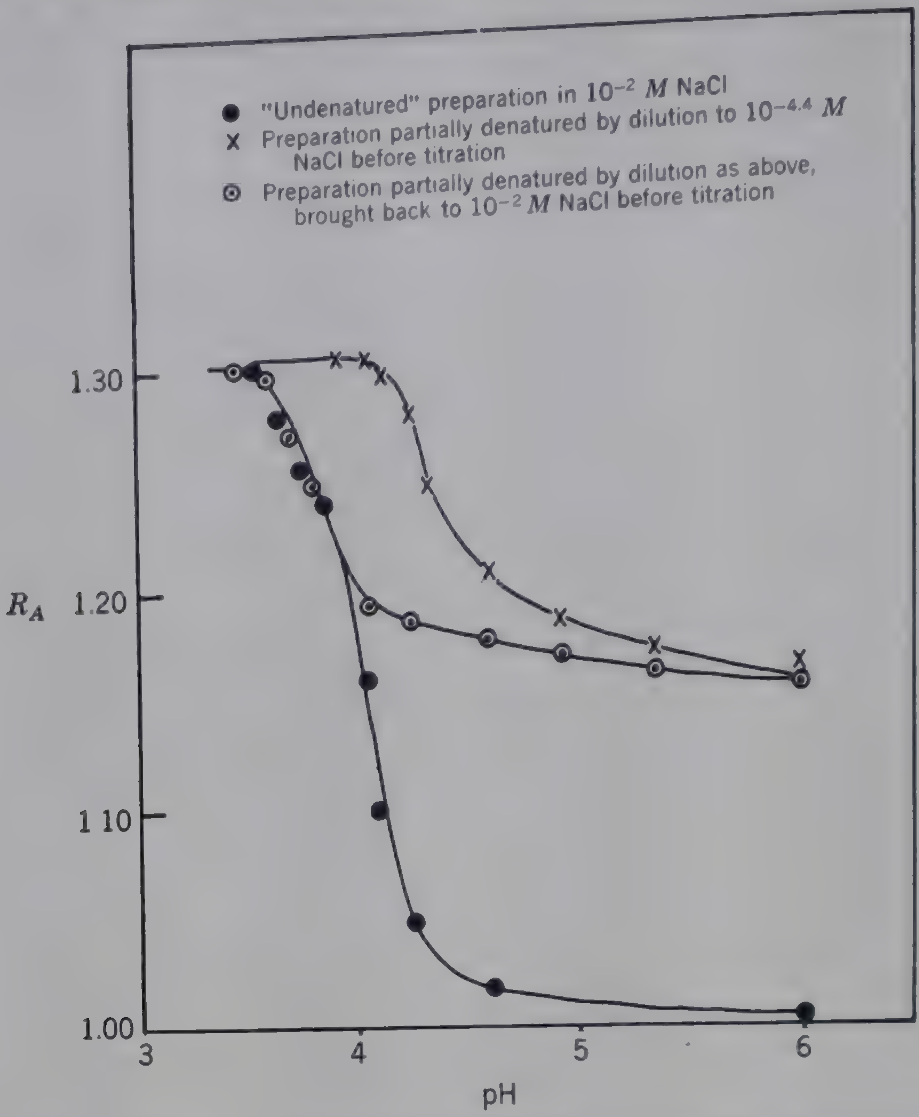

FIG. 26. The variation of ϵ(P) with pH of "undenatured" and partially denatured preparations of calf thymus DNA (Thomas[78]).

The ordinate scale is the ratio, R_A, of the observed ϵ(P) value to the minimum value (measured at pH 6 in 10^{-2} M NaCl solution). The titration in each case is carried out from the higher pH value.

denaturation in such solutions depends on the time lapse between the preparation of the solution and examination.

A further protective effect of salts against heat denaturation of DNA solutions has been observed by Thomas.[78,81] For calf thymus DNA in sodium chloride solutions of various concentrations, some denaturation occurs at room temperature at $<10^{-4}$ M and below 70° in 10^{-2} M. Denaturation is strongly inhibited even at 100° C. in 10^{-1} M and M solutions. It is claimed that heat denaturation occurs stepwise and that the critical temperature for each step varies for DNA from different sources. Thus DNA from starfish testis in 10^{-2} M sodium chloride undergoes the first step in denaturation at 55° C. compared with 70° C. for that from calf thymus.

The bearing of these results on theories of the structure of DNA may become apparent when they are correlated with the study of other physical properties. This may not be a simple matter because of the very different nucleic acid concentrations at which spectrophotometric and other physical

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[81] R. Thomas, *Trans. Faraday Soc.* **50**, 290 (1954).

measurements are made. The concentrations of DNA required for electrometric, conductimetric, and viscosimetric measurements may be one hundred times greater than those required for spectrophotometric measurements.[53,82]

5. Effect of Other Agents on the ε(P) of DNA

Thomas[81] has shown that urea (6 *M* at 20° C.) is without denaturing effect on DNA. Blout and Asadourian,[75] working with a DNA stock solution in distilled water, have found that urea lowers ε(P) slightly, whereas plasma albumin and lysozyme have a marked effect in the same direction. They also find that non-ionic compounds such as polyvinyl alcohol, sucrose, and glycerol have no effect even in high concentration. It appears, however, from Lawley's results[56] that ethanol increases ε(P): it is maximally effective in 50% concentration while at higher or lower concentrations ε(P) approaches its distilled water value. He has also shown that Ag^+ has a specific action on DNA, unlike that of other metal cations. Addition of silver nitrate to a solution of DNA first lowers the ε(P) value, this lowering being maximal for one equivalent of Ag^+ per 4 atoms of DNA phosphorus. Further addition causes a restoration of ε(P) to the value for the DNA in distilled water. The explanation for this is still obscure, but Lawley suggests that the bases themselves are involved. It would be of interest to examine the action of other heavy metals on the ε(P) value of a nucleic acid. Such measurements will be limited in many cases by the insolubility of the products.[83,84]

6. Spectrophotometric Estimation of Nucleic Acids

In general the spectrophotometric analysis of nucleic acids is carried out by hydrolysis to free nucleotides, nucleosides, or bases, followed by their separation and individual estimation, from which an estimate of the total nucleic acid may be obtained. [Cf. Chapters 5–7, 9–11.]

However, methods for direct estimation of nucleic acids by spectrophotometric means have been developed. Ogur and Rosen[61] by differential extraction with perchloric acid have estimated DNA and PNA in corn root tips and rabbit liver in this way. Tsuboi[63] and Logan *et al.*[85] have used trichloracetic acid in a similar method. These procedures cause considerable degradation of the nucleic acids which, however, does not result in any net loss of absorbing material. It is, in fact, an essential feature of these methods that the nucleic acids should be degraded so that the ε(P) anomaly

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[82] J. A. V. Butler and B. E. Conway, *Nature* **172,** 153 (1953).

[83] E. Hammarsten, *Biochem. Z.* **144,** 383 (1924).

[84] K. G. Stern and M. A. Steinberg, *Biochim. et Biophys. Acta* **11,** 553 (1953).

[85] J. E. Logan, W. A. Mannell, and R. J. Rossiter, *Biochem. J.* **51,** 480 (1952).

is completely abolished, thus enabling a direct correlation between al sorptivity and concentration to be made. It is evident from Ogur an Rosen's analyses that the ε(P) of yeast PNA from different sources, afte such degradation, is remarkably reproducible, having a value of 10.80 (in *N* perchloric acid) with a standard deviation of 1.3 %. Their corresponding figure for thymus DNA is 8,780, with a standard deviation of 2.3 % Such values of ε(P) will vary with the purine and pyrimidine contents o the particular nucleic acid, so that in order to apply the method generall this ε(P) value must be established in each case. [Cf. Chapter 10; Chapte 11.]

7. Nucleoproteins

The ultraviolet absorption spectrum of a nucleoprotein is composed c contributions from both constituents and therefore differs from that of th nucleic acid alone. The protein absorption,[86] which depends mainly on it content of aromatic amino acid residues, is maximal at *ca.* λ 280 mμ, an rises again at shorter wavelengths from a minimum at *ca.* λ 250 mμ. On weight basis the peak absorption of protein will be only about one-tenth c less that of nucleic acid, so that for a nucleoprotein containing, say, 40 % of nucleic acid, the absorption contribution of the latter therefore dominate the collective absorption curve, and the contribution of the protein usuall appears as an inflection at *ca.* λ 280 mμ while the nucleic acid minimum a *ca.* λ 230 mμ is shifted to longer wavelength. Since the nucleic acid conten of nucleoproteins varies over a wide range (e.g., 5–40 % for some plan viruses,[87] while the absorption of some of the proteins associated witl nucleic acids tends to be very low, the spectra of nucleoproteins will ob viously vary between rather wide limits; many examples can be found i the literature.[88,89]

Various suggestions have been made regarding the possible mode of combinatio of the nucleic acid and protein moieties of nucleoprotein. Astbury[90] pointed out tha the *ca.* 3.4-A. X-ray diffraction spacing of nucleic acids was comparable with th side-chain spacing of a fully extended β-type polypeptide chain and suggested tha this correspondence might allow salt-like electrostatic bonds to be formed betwee the phosphate groups of the Astbury polynucleotide chain model and the guanidin groups of arginine side chains, which form a large proportion of the amino aci residues in basic proteins of the histone and protamine types. Support for this viev comes from the analytical data of Vendrely and Vendrely,[91] who find a constant DNA–arginine ratio in the erythrocyte nuclei of many fish species which show large variations in DNA content. Some interesting observations on the composition and solubility of synthetic DNA–protamine complexes in relation to natural nuceloproteins are discussed by Alexander.[92] Stern[93] has found that model polynucleotide and polypeptide chains can interlace without straining of bond angles to allow the formation of salt linkages, as envisaged by Astbury, in such a way as to lock the polynucleotide chain in a specific configuration. In this connection it should perhaps be noted that free DNA preparations from different species show the same structure independent of the base constitution.[94]

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[86] G. H. Beaven and E. R. Holiday, *Advances in Protein Chem.* **7,** 319–386 (1952).

[87] J. P. Greenstein, *Advances in Protein Chem.* **1,** 209 (1944).

[88] R. Markham, R. E. F. Matthews, and K. M. Smith, *Nature* **162,** 88 (1948).

[89] T. Caspersson, "Cell Growth and Cell Function." Norton, New York, 1951 *Symposia Soc. Exptl. Biol.* **1,** 127–151 (1951).

[90] W. T. Astbury and F. O. Bell, *Nature* **141,** 747 (1938); see also W. T. Astbury *Symposia Soc. Exptl. Biol.* **1,** 66–76 (1951).

Riley and Arndt[95] have concluded from quantitative X-ray scattering studies of herring sperm and calf thymus nucleoproteins and of the constituent nucleic acids and proteins (clupeine and histone, respectively) that a nucleoprotein is best regarded as a fairly gross addition complex, presumably held together by salt linkages, rather than an intimate and specific association of the two components, for example, a fully extended β-type polypeptide chain wound around the double-helix polynucleotide chain, as envisaged by Watson and Crick.[77]

It is not yet possible, on the basis of these conflicting views on the nature of the protein–nucleic acid association, to make any prediction about the additivity or otherwise of the absorptivity contributions of the two components of a nucleoprotein. Some experimental data are available. Shack and co-workers[70,96] find that the anomalous spectrophotometric titration of thymus DNA is also shown by the related nucleohistone and by one isolated from a transplantable mouse lymphoma. They conclude that the same alkali-labile hydrogen-bonded structure is present in a nucleoprotein and the derived nucleic acid.

Blout and Asadourian[75] have measured the absorbance of calf thymus DNA solutions containing added protein differentially against control solutions of protein and find that both lysozyme and bovine plasma albumin lower the DNA absorbance; in the presence of sodium chloride, the lowering is additional to the effect of the salt alone. These workers recognize three factors which determine the absorptivity of a given solution of DNA, (1) pH, (2) a "small ion" effect, and (3) a "macromolecular ion" effect. The first two factors have been discussed above; the third is supposed by Blout and Asadourian to operate in the same was as (2), viz., by decreasing the interaction (of unspecified character) between neighboring units in the polynucleotide chain. Non-ionic macromolecular substances such as polyvinyl alcohol are without effect.

Brachet is reported [97] to have evidence that the absorption of mixtures of thymus DNA and lysozyme (but not histone) is not additive.

Seibert[98] has found deviations from additivity for the total absorbance of mixtures of tuberculin proteins and thymus DNA over a wide range of pH. At low pH valu the measurements are complicated by opalescence, but above pH 5 the differenc between the calculated and measured mixture absorbances are much greater tha could reasonably be ascribed to spectrophotometric errors, and indicate that t total absorbance is reduced in the mixture, deficits of 10% or more being observed.

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[91] R. Vendrely and C. Vendrely, *Nature* **172,** 30 (1953).

[92] P. Alexander, *Biochim. et Biophys. Acta* **10,** 595 (1953).

[93] K. G. Stern, The chemistry and physiology of the nucleus, *Exptl. Cell Research,* Suppl. 2 (1952).

[94] M. H. F. Wilkins, A. R. Stokes, and H. R. Wilson, *Nature* **171,** 738 (1953).

[95] D. P. Riley and U. W. Arndt, *Nature* **172,** 294 (1953).

[96] J. Shack and J. M. Thompsett, *J. Natl. Cancer Inst.* **13,** 1425 (1953).

[97] H. G. Davies and M. P. B. Walker, Microspectrometry of living and fixed cells, *Progr. Biophys. and Biophys. Chem.* **3,** 195–236 (1953).

[98] F. B. Seibert, The physical chemistry of proteins, *Discussions Faraday Soc.* **13,** 251 (1953).

The limited experimental work on this subject does not reveal whic component of a nucleic acid–protein system gives rise to the absorbanc deficit, or if both components are affected. In particular it is not yet know if combination with a protein has the same effect as Mg^{++} or Na^+ ions (i appropriate concentrations) in restoring the low $\epsilon(P)$ value characterist of the native state to a DNA preparation that has previously been partl denatured (with consequent increase in $\epsilon(P)$) by exposure to low salt concentration, etc. Clearly there is scope for accurate spectrophotometri studies on nucleoproteins in relation to the absorption properties of the constituent proteins and nucleic acids and the effects of denaturation.

IV. Ultraviolet Dichroism

The dichroism of the ultraviolet absorption of oriented fibers and sheet of high-molecular-weight nucleic acid may be used to obtain informatio concerning the orientation of the purine and pyrimidine rings with respec to the long axis of the macromolecule. This axis may be taken as parallel t the fiber axis or direction of shear in specimens which have been oriente by stretching or shearing, respectively. Dichroism measurements may als be used to study the state of nucleoprotein as it exists in intact cells. Th possibility that the dichroism of nucleic acid in biological material ma give rise to serious errors in the microspectrometric estimation of nuclei acid has been the subject of much recent comment. For these reasons i has seemed advisable to review briefly the present status of dichroisn studies, although it must be emphasized that a full treatment would requir an extensive development of the underlying principles of crystal optics Here it is only possible to give references to some standard texts on th subject and to state the appropriate concepts and equations without detailed exposition.[99-102]

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[99] H. Ambronn and A. Frey, "Das Polarisationsmikroskop." Akad. Verlagsgesellschaft, Leipzig, 1926.

[100] C. W. Bunn, "Chemical Crystallography," 1st ed. Oxford University Press London, 1945.

[101] N. H. Hartshorne and A. Stuart, "Crystals and the Polarising Microscope, 2nd ed. Edward Arnold, London, 1950.

[102] E. E. Jelley, Microscopy, *in* "Physical Methods of Organic Chemistry," Vol. 1 Part 1 of "Technique of Organic Chemistry," (A. Weissberger, ed.), 2nd ed. Interscience Publishers, New York, 1949.

1. General Theory

The nature of an electronic transition in a molecule caused by the absorption of radiation of appropriate frequency requires that for maximum probability the electric vector of the radiation should have a definite direction with respect to the chromophoric group of the molecule. For a linear conjugated system, such as a polyene, the electric vector must be parallel to the direction of the conjugated multiple bonds, while for a planar conjugated system, of which benzene is the simplest example, the electric vector must be in the plane of the ring. The absorption is greatest when these conditions are satisfied. If the electric vector is perpendicular to the plane of the ring the transition probability, and therefore the intensity of absorption, is reduced, though not necessarily to zero, because of interaction between the two modes of excitation. These polarization properties apply both to allowed transitions and also to the much weaker transitions, e.g., that giving rise to the 260-mμ benzene band, which acquire allowed character because of the disturbing effect of simultaneous changes in the vibrational states. If the ring chromophore is unsymmetrical, the strength of the transition may also depend on the direction of the electric vector in the plane of the ring.

The intensity of absorption of polarized radiation by a crystal or oriented specimen will therefore depend on the orientation of the electric vector of the radiation with respect to the absorbing groups of the constituent molecules, and hence in the case of a crystal, with respect to the crystallographic axes. For an oriented fiber or sheet, the axis of reference will be the fiber axis or shear direction, as noted above. The crystal or specimen will therefore require more than one absorptivity to express its absorption properties, and may be described as dichroic (or trichroic if three such coefficients are required). These are known as the principal absorptivities and can be referred to specific directions with the crystal or system. Since the anisotropy of refractive index (birefringence) for unabsorbed light in a region of transparency is also a consequence of the molecular orientation of the specimen, the directions of the principal refractive indices, and hence of the extinction directions, will be related to, and for uniaxial orientation, will coincide with, the directions of the principal absorptivities. The birefringence and dichroism are not necessarily parallel indications of the orientation of the same groups in a molecule since in a region of transparency the birefringence is the sum of contributions by all the bonds in the molecule whereas in an absorbing region the dichroism is a characteristic of the absorbing groups only.

Absorption measurements in solution refer to randomly oriented molecules (except for the special case of solutions of long-chain macromolecules oriented by flow) in which the principal absorption directions are in a possible directions with respect to the electric vector of the (nominally unpolarized incident radiation. In order to measure the principal absorptivities it is necessary to use crystals or oriented specimens and plane-polarize radiation. Ideally, the arrangement of the molecules in a crystal with respect to the crystallographic axes should be known from an X-ray structur determination, but even if this information is lacking the dichroism alon can be used to obtain information about the probable orientation of th absorbing groups within the crystal.

In practice absorption measurements on crystals or oriented materia usually entail the use of very small specimens. This limitation arises partl because of the difficulty in preparing large crystals or specimens (or th inherent size of specimens of biological origin), and partly because of th high absorbances that would be encountered with thick samples. For thi reason, it is necessary to use some form of fully achromatic reflecting microscope in conjunction with the spectrophotometric equipment. With regar to crystals, the number of compounds in which the arrangement of th molecules within the crystal lattice is known from X-ray structure data t be favorable for the study of pleochroism is very small. The total volum of work in the field is therefore limited, though the introduction of variou designs of reflecting microscope has led to increased activity.

Seeds[103] has reviewed the polarized ultraviolet microspectrography o crystals and oriented systems of biological interest, and this work shoul be consulted for experimental details. General microspectrophotometr has been dealt with in considerable detail by Seeds, Wilkins, Barer, Davies, Mellors, Walker, Commoner, and other contributors to a recent symposium[104] which contains much information on reflecting optics and microspectrophotometric systems, together with typical results on a wide variet of biological material.

2. Form Dichroism

Form anisotropy, both birefringence and dichroism, is encountered in composite systems containing oriented assemblages of macromolecules, even if the latter are themselves isotropic, when the systems contain amorphous regions between the ordered ones. The form anisotropy is additional to any intrinsic anisotropy that the oriented macromolecules may exhibit and can therefore make an important contribution to the total observed anisotropy of a system in which the conditions for form anisotropy are satisfied. The two models which have been treated theoretically by Wiener[105] are isotropic rods arranged in parallel bundles and flat plates in parallel stacks, separated in both cases by an amorphous component of different refractive index. Wiener's equations apply strictly to systems in which the volume concentration of the ordered phase is small and is therefore not accurate for, e.g., a slightly swollen fiber, in which the ordered phase is a large proportion of the total volume. It has been stated[106] that a more accurate theory for hexagonally packed rods is being prepared. Examples of the use of the Wiener formulas have been given by many authors[107-109] in connection with the optical properties of natural cellulosic fibrous structures.

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[103] W. E. Seeds, Polarized ultraviolet microspectrography and molecular structure, *Progr. Biophys. and Biophys. Chem.* **3,** 27–46 (1953).

[104] Spectroscopy and molecular structure and optical methods of investigating cell structure, *Discussions Faraday Soc.* No. **9** (1950).

Form anisotropy can be detected by changing the refractive index of the amorphous component (by immersion in liquids of various refractive indices) and observing the effect on the birefringence. At a particular refractive index of the immersion liquid the form birefringence (positive for rod model, negative for lamellar model) falls to zero, and any residual birefringence is then intrinsic, due to anisotropy of the macromolecules in the ordered phase.

A good example of this behavior was given by Weber[110] (quoted by Doty and Geiduschek[111]) for artificial gelatin and myosin fibers; both showed positive birefringence, but, at the critical refractive index, the residual birefringence of the gelatin fiber was zero, while for the myosin fiber a large intrinsic birefringence persisted. Some observations on nucleoproteins and nucleic acids in which form anisotropy may have been involved will be noted later in this section.

3. DEFINITIONS AND EQUATIONS[103]

A linear absorbing system is characterized by two absorptivities, $a_\parallel$ and $a_\perp$ for the electric vector parallel and perpendicular, respectively, to the direction of the absorbing system. Then

$$\text{Dichroism} = a_\parallel - a_\perp$$

$$\text{Dichroic Ratio } (D) = a_\parallel / a_\perp$$

In general a biaxial crystal will have three absorbances (A_x, A_y, A_z) corresponding to the principal orthogonal axes of the triaxial absorption ellipsoid. These are related to the non-oriented value (A') for the sam compound by the equation

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[105] O. Wiener, *Abhandl. math.-phys. Kl. sächs. Akad. Wiss. (Leipzig)* **32,** 507 (1912); *Kolloidchem. Beih.* **23,** 189, 198 (1926).

[106] M. H. F. Wilkins, A. R. Stokes, W. E. Seeds, and G. Oster, *Nature* **166,** 127 (1950).

[107] F. O. Schmidt, *Advances in Protein Chem.* **1,** 25–68 (1944).

[108] A. Frey-Wyssling, "Submicroscopic Morphology of Protoplasm," Elsevier, Amsterdam, London, New York, Houston, 2nd English Ed., 1953.

[109] A. Frey, *Jahrb. wiss. Botan.* **67,** 597 (1927).

[110] H. H. Weber, *Pflügers Arch. ges. Physiol.* **235,** 205 (1934).

[111] P. Doty and E. P. Geiduschek, *in* "The Proteins" (H. Neurath and K. Bailey, eds.), Vol. 1, part A. Academic Press, New York, 1953.

$$A' = (A_x + A_y + A_z)/3 \qquad \text{(biaxial case)}$$

or

$$A' = (2A_{\perp} + A_{\|})/3 \qquad \text{(uniaxial case)}$$

The calculated value of A' can be used to estimate the thickness of th crystal specimen by applying the Beer-Lambert absorption laws to solutio data for the same compound, assuming that the specific absorptivity (a o ϵ) is identical in the crystal and in solution (with identical concentratio units).

Except in certain favorable cases (e.g., hexamethylbenzene, see below the absorbing groups in crystals and oriented materials are not all paralle to each other, but may be in arrangements in which the dichroism of on set of parallel groups is neutralized, to a greater or lesser extent, by th opposing dichroism of other sets of parallel groups. Consequently the observed dichroic ratio is often much smaller (2 or less) than would be expected from simple theory. As emphasized by Seeds[103] the interpretatio that can be given to such low dichroic ratios depends on making a choic between two assumptions. The system under study can be assumed to b partly disoriented, so that the observed low dichroism is taken as som measure of the degree of orientation. Such an assumption is obviously not valid for crystals proper, for which, in any case, X-ray data might be available to indicate that the crystal lattice is unfavorable. For artificially oriented materials of natural or synthetic origin, imperfect orientation i much more likely, although errors arising from it can be minimized i microspectrographic procedures by confining the measurements to th specimen areas with the highest birefringence. It may then be assumed that the specimen is fully oriented and that the observed dichroism is a measure of the average angle of tilt of the absorbing groups in the molecul to the principal axis of the specimen. If it is further assumed that the dichroic ratio for a planar ring is very large and that dichroism in the plan of the ring is small, the variation of D with angle of tilt for some simple model structures can be calculated.

For the important practical case of a uniaxial fiber with rotational symmetry, in which the normals to the planes of the absorbing groups lie on a cone of semi-angle θ generated about the fiber axis, Fraser[112] has shown that

$$D_{\text{fiber}} = \text{Sin}^2\,\theta/1 - \tfrac{1}{2}\,\text{Sin}^2\,\theta$$

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[112] R. D. B. Fraser, Ph. D. Thesis, London University, 1951, quoted by Seeds.[103]

For oriented sheets

$$D_{\text{sheet I}} = \text{Sin}^2\,\theta/\text{Cos}^2\,\theta$$

$$D_{\text{sheet II}} = \text{Sin}^2\,\theta$$

Sheet model type I is for the case in which the normal to the planes of the absorbing groups makes an angle θ with the direction of shear and lies in a plane normal to the direction of the incident radiation. In the type II model the normal to the absorbing planes lies at an angle θ to the direction of shear and in the plane of incidence of the radiation.

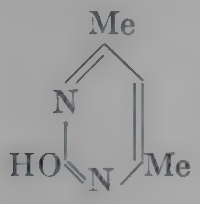
By using these equations some indication of the mean orientation of the absorbing groups can be obtained. Since observations on crystals with favorably disposed absorbing groups indicate that absorption is not vanishingly small when the electric vector is perpendicular to the ring plane, these simple relationships have to be modified if it is required to estimate θ from very large or very small dichroic ratios.

4. Dichroism of Simple Compounds

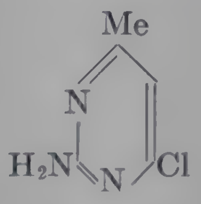
A much-quoted demonstration of the dichroism of the benzene chromophore is due to Scheibe, Hartwig, and Müller using hexamethylbenzene.[113] This compound crystallizes with all the planar molecules parallel to one another and can be obtained as crystalline sheets or sublimed crystalline films in which the planes of the benzene groups are roughly normal to the specimen plane. Such a preparation has a dichroic ratio of *ca.* 10 over most of the absorption band with $\lambda_{\text{max.}}$ *ca.* 270 mμ (corresponding to the well-known "forbidden" benzene band at *ca.* 255 mμ). Scheibe *et al.* also find that the wavelengths of the vibrational fine-structure bands are slightly different for the electric vector parallel and perpendicular to the ring planes.

Craig and Lyons[114] have extended these observations to the 230-mμ band, which is also polarized in the molecular plane, the dichroic ratio for this band being of the same order. This result, which is applicable by analogy to the benzene "allowed" system of *ca.* 200 mμ, is of great importance for current theoretical arguments about the assignment of the benzene 200-mμ system and the nature of the molecular orbitals involved.

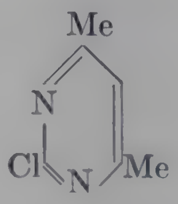
Dichroism studies on the particular pyrimidines and purines found in nucleic acids have not yet been reported, probably because of difficulty in obtaining suitable crystal specimens and the lack of X-ray structure data. Seeds[103,115,116] has studied 2-hydroxy-4,6-dimethylpyrimidine (I) for which favorable X-ray data are available.



I



II



III

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[113] G. Scheibe, St. Hartwig, R. Müller, *Z. Elektrochem.* **49,** 372 (1943).

[114] D. P. Craig and L. E. Lyons, *Nature* **169,** 1102 (1952).

[115] W. E. Seeds, Ph.D. Thesis, London University, 1951.

[116] W. E. Seeds, *Discussions Faraday Soc.* No. **9,** 394 (1950).

Absorption measurements with the electric vector in and perpendicular to the rin plane show very marked dichroism; D approaches 100 at 300 mμ where the perpendicular absorption is very small, while the in-plane absorption extends to a much longe wavelength. The dichroism decreases with decreasing wavelength and at 250 mμ has fallen to *ca.* 2. By a special crystallization method Seeds has also been able t make observations with the electric vector in the ring plane and parallel or perpendicular to the pseudo-dyad axis through C_2–C_5 and finds a small but definite dichroism below 300 mμ.This work appears to be the first in which the three absorption spectra of a crystal have been measured and dichroism in the ring plane demonstrated Craig and Lyons[117] have since demonstrated that in naphthalene the transitio dipole moment for the weak transition at *ca.* 310 mμ lies predominantly in the molecular plane and along the shorter molecular axis.

Lyons[118] has examined 2-amino-4-chloro-6-methylpyrimidine (II), for which X-ra structure data are available, and 2-chloro-4,6-dimethylpyrimidine (III). Of the thre band systems generally shown by pyrimidines[29] the weak longwave band (a) is obscured (though not in III) by system (b) located at 250–290 mμ; the third system (c lies at 200–250 mμ. Band (a) disappears in acidic solution and is a transition of th n–π type[119] involving non-bonding electrons on the nitrogen atoms while bands (b and (c) are due to π–π transitions corresponding to the 260-mμ and 200-mμ benzen systems. For the type (b) band of II the electric vector is in the molecular plane fo strong absorption in agreement with the results for I and there is a favored directio (x) in the plane parallel to a line through the two nitrogen atoms. For III, with unknown crystal structure, the polarization of bands (a) and (c) are similar and differen from that of band (b). Since by analogy with hexamethylbenzene band (c) of II an III will be polarized in the molecular plane, their polarization direction in the plan must be mainly y, at right angles to that of the (b) band. From these findings Lyon is able to give assignments to the type (b) and (c) systems and to draw some importan conclusions about the nature of the upper state of the type (a) n–π transition.

5. Dichroism of Nucleic Acid

The optical anisotropy of biological materials is the subject of an extensive literature (see review[120]) mainly concerned with birefringence studies. Sperm heads have long been known[121] to show a large optical anisotropy, indicating parallelism of the nucleoprotein molecules, and it ha very recently been shown by X-ray diffraction studies that this anisotrop arises from a true three-dimensional crystallinity.[122] The birefringence o cytoplasm has been the subject of much work, but it has recently bee pointed out[120] that form and intrinsic birefringence have not always bee distinguished, while the sign of the birefringence is certainly not sufficien to distinguish oriented protein from oriented nucleic acid. The high[122a] negative birefringence of extracted high-molecular-weight thymus DNA solution, oriented by flow, was immediately interpreted by Signer, Caspersson, and Hammarsten[123] as indicating that the absorbing groups were oriented with their ring planes perpendicular to the long axis of the macromolecule, and this conclusion was utilized by Astbury and Bell[90] in their model of the DNA molecule in which the nucleotide residues were stacked parallel to each other along one side of the long molecular axis. [Cf. *Jordan*, Chapter 13.]

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[117] D. P. Craig and L. E. Lyons, *J. Chem. Phys.* **20,** 1499 (1952).

[118] L. E. Lyons, *J. Chem. Phys.* **20,** 1814 (1952).

[119] M. Kasha, *Discussions Faraday Soc.* No. **9,** 14 (1950).

[120] M. M. Swann and J. M. Mitchison, Birefringence of cytoplasm and cell membranes *Progr. Biophys. and Biophys. Chem.* **2,** 1–16 (1951).

[121] W. J. Schmidt, "Die Doppelbrechung von Karyoplasma, Zytoplasma und Metaplasma." Borntraeger, Berlin, 1937.

[122] M. H. F. Wilkins and J. T. Randall, *Biochim. et Biophys. Acta* **10,** 192 (1953).

The negative ultraviolet dichroism of a thymus DNA film oriented by stretching was first demonstrated by Caspersson,[124] whose results show a dichroic ratio at the 260-mμ absorption maximum of *ca.* 1.6 and also good agreement between the observed absorption curve for unpolarized light and that calculated from the values obtained with the electric vector parallel and perpendicular to the direction of stretch. No dichroism was observed with yeast PNA films, in agreement with indications from other techniques that this material was unoriented. It has usually been considered that because of its lower molecular weight and possibly because of structural differences, PNA will not show molecular orientation to the striking extent found with DNA. However, PNA from calf and rat liver, prepared with precautions against depolymerization,[125] shows flow birefringence (about one-tenth of the value for highly polymerized DNA) and forms birefringent fibers which are elastic when undried (cf. sodium DNA fibers, see below). It seems likely that, when the problems of preparing undenatured PNA have been solved, this class of nucleic acids will also lend itself to studies of optical anisotropy.

Seeds[103,115,126] has obtained dichroic ratios of 1.7–2 for air-dried (*ca.* 30% water) films of thymus DNA oriented by shearing a viscous gel, the dichroism being roughly constant over the entire absorption band. Observations over a range of controlled humidity show that the dichroism increases with increasing humidity up to 90%, above which the specimen becomes unstable to irradiation and changes eventually to an isotropic form. The highest dichroic ratio recorded by Seeds is 4.7 at the 265-mμ maximum for a film in air at 93% humidity; the average value for air-

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[122a] For oriented fibers with rotational symmetry about the long axis and for their films oriented by shearing, etc., the sign convention for birefringence and dichroism is that a positive fiber has its greater refractive index (or absorbance) along the fiber length. When the greater refractive index (or absorbance) is perpendicular to the fiber axis the sign is negative, as in nucleic acid. (Cf. ref. 101, pp. 148, 441).

[123] R. Signer, T. Caspersson, and E. Hammarsten, *Nature* **141,** 122 (1938).

[124] T. Caspersson, *Chromosoma* **1,** 605 (1940); cf. J. P. Greenstein, ref. 87.

[125] E. L. Grinnan and W. A. Mosher, *J. Biol. Chem.* **191,** 719 (1951).

[126] W. E. Seeds and M. H. F. Wilkins, *Discussions Faraday Soc.* No. **9,** 417 (1950).

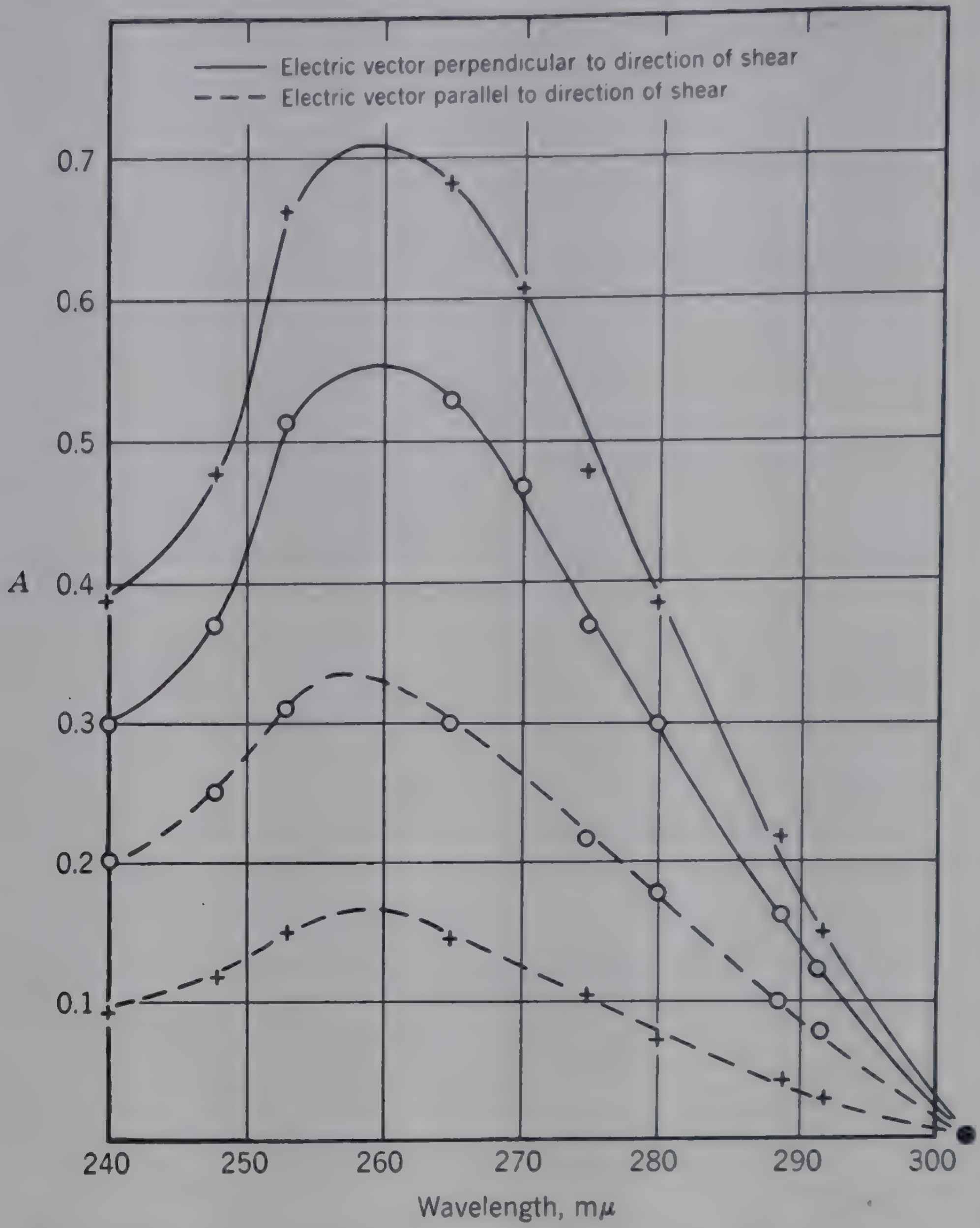

FIG. 27. Ultraviolet absorption spectra of oriented film of thymus DNA sodium salt: (+) at 90% humidity, (○) at 60% humidity (Seeds [103, 115]).

dried oriented DNA is *ca.* 2, increasing to nearly 4 at 90% humidity. (Fig. 27.) The change in dichroic ratio with humidity can be interpreted, using the equations for model systems as a rotation of the planes of the absorbing groups, the angle obtained depending on whether the uniaxial fiber or type II sheet model is selected. Seeds concludes that the bases are so arranged that, on the average, they lie at a small angle to the normal to the molecular axis and that this angle decreases with increasing humidity; the actual structure is probably intermediate between the two model structures considered.

The influence of humidity on the anisotropy of oriented DNA fibers has been studied in greater detail by Wilkins, Gosling, and Seeds,[127] who recognize two distinct forms with strikingly different properties:

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[127] M. H. F. Wilkins, R. G. Gosling, and W. E. Seeds, *Nature* **167**, 759 (1951).

Type I (stable at high humidity) with:
Negative birefringence
Negative ultraviolet dichroism
Mainly negative infrared dichroism
X-ray diffraction pattern of well-ordered crystallites
Type II (stable at 50% humidity) with:
Positive birefringence
No ultraviolet dichroism
Mainly positive infrared dichroism
Diffuse-X-ray diffraction pattern

Type II can be obtained from a type I fiber by stretching up to nearly 100% elongation in air of 50% relative humidity. Reconversion to type I, with appreciable contraction, occurs on exposure to higher humidity. Wilkins *et al.* consider that the dimensional change associated with hydration is due to conversion from amorphous to the crystalline state and not to water molecules packing between the crystallites. On dehydration it is suggested that the molecular backbone of phosphate ester linkages crumples and the bases tilt with consequent destruction of the ultraviolet dichroism (and reversal of birefringence), while in the hydrated I-form the extended molecules pack together regularly with the planes of the bases approximately at right angles to the long axis; in the II-form the rings are estimated to lie at an average angle of *ca.* 45° to the fiber length. Additional information on the difference between the I- and II- forms of DNA is given by Franklin and Gosling[128] in a discussion of X-ray diffraction evidence supporting the Watson and Crick[77] double coaxial helical structure for sodium-DNA. They consider that in the highly ordered I-form at 75% humidity the planes of the base rings are at an angle of *ca.* 25° to the fiber axis; this value, and that estimated by Wilkins *et al.*[127] for the II-form, appear to be consistent with the changes in dichroism observed for the I $\rightleftharpoons$ II interconversion process.

It is evident that in these structural studies the ultraviolet dichroism has provided valuable supplementary information to the X-ray diffraction and other optical investigations. [Cf. *Jordan*, Chapter 13.]

Work has been reported on the ultraviolet dichroism of the nucleoprotein (containing PNA) associated with tobacco mosaic virus. Butenandt and co-workers[129] measured the dichroism of solutions oriented by streaming, and their essential results have been confirmed by Seeds and Wilkins[103,115,126] working with both flow-oriented solutions and shear-oriented gels, who find, however, the dichroism to be of opposite sign from that reported by Butenandt *et al.*, i.e., absorption is greatest with the electric vector parallel to the flow (or shear) direction. This alteration in sign is confirmed by Perutz, Jope, and Barer,[130] using the microspectrographic technique of Barer *et al.*,[131] adapted for work with polarized radiation. These three groups of workers are mainly concerned with the significance to be attached to the parallel dichroism of the tryptophan fine-structure band at *ca.* 290 mμ of the protein moiety. Seeds and Wilkins reject the conclusion of Butenandt *et al.* that the dichroism of similar sign and magnitude in the 260-mμ region is an indication of orientation of the nucleic acid, and suggest that it is due to form dichroism. The optical properties of the hexagonal crystals present in the leaf hairs and other cells of plants infected with tobacco mosaic virus have also been studied,[106] but the results obtained are too complex to be reviewed here. Calf thymus nucleoprotein (containing DNA) gives oriented films and fibers (though less readily than the derived DNA) which are negatively birefringent. As with nucleic acid, the ultraviolet dichroism is negative, with maximum absorption when the electric vector is perpendicular to the direction of shear, and dichroic ratios of 1.5–1.7 have been observed for air-dried specimens of birefringence *ca.* −0.014.[103,126]

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[128] R. E. Franklin and R. G. Gosling, *Nature* **171,** 740 (1953); **172,** 156 (1953).

[129] A. Butenandt, H. Friedrich-Freksa, St. Hartwig, and G. Scheibe, *Z. physiol. Chem.* **274,** 276 (1942).

6. INFLUENCE OF DICHROISM ON THE MICROSPECTROGRAPHIC ESTIMATION OF NUCLEIC ACID IN INTACT CELLS

The use of a refracting microscope for the ultraviolet microspectrometry of intact cells, was initiated by Caspersson[132] in 1936 and has since been intensively developed[133] and exploited[89] by him and his co-workers as a valuable cytochemical tool. [Cf. *Swift*, Chapter 17.] More recently, fully achromatic reflecting microscopes have been devised for such work; the results obtained have been reviewed by Davies and Walker,[97] and instrumental problems elsewhere [104]

Commoner and Lipkin[134] suggest that orientation, which is known from birefringence observations to be often present to a greater or lesser extent in biological material, may be expected to invalidate absorption measurements with unpolarized light because of Beer-Lambert absorption law deviations which are, according to their arguments, inherent in such systems. Their views have been the subject of much comment.

Thorell and Ruch[135] have measured the absorption at 260 mμ of sodium-

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[130] M. F. Perutz, E. M. Jope, and R. Barer, *Discussions Faraday Soc.* No. **9**, 423 (1950).

[131] R. Barer, E. R. Holiday, and E. M. Jope, *Biochim. et Biophys. Acta* **6**, 123 (1950).

[132] T. Caspersson, *Skand. Arch. Physiol.* **73**, Suppl. 8 (1936).

[133] T. Caspersson, *Exptl. Cell. Research* **1**, 595 (1950); T. Caspersson, F. Jacobsson, and G. Lomakka, *ibid.* **2**, 301 (1952).

[134] B. Commoner, *Science* **110**, 31 (1949); B. Commoner and D. Lipkin, *ibid.* **110**, 41 (1949).

[135] B. Thorell and F. Ruch, *Nature* **167**, 815 (1951).

DNA solutions oriented by streaming and of gels oriented by stretching, both with unpolarized radiation and with radiation polarized parallel and perpendicular, respectively, to the orientation direction. Their theoretical analysis indicates that the error in absorption measurements on such oriented systems with unpolarized radiation will be dependent on the dichroic ratio, and they suggest that the magnitude of the error must be investigated experimentally for each particular case. Their results show small errors, *ca.* 5%, for specimens with quite high dichroic ratios (up to 3) and high transmission, but the error is very much larger for low transmissions. They conclude, however, that the dichroism of the nucleic acid in cytological material is so low, except for material such as sperm heads, that the effect of molecular orientation on the absorption measured with unpolarized light is unlikely to introduce the serious errors adduced by Commoner and Lipkin.

As noted above, Caspersson's early ultraviolet absorption measurements[124] on an oriented film of DNA show good agreement between the curve for unpolarized radiation and the average of the two curves for radiation polarized parallel and perpendicular to the orientation axis. In this example the average absorbance is *ca.* 0.6 (approx. 25% transmission) and the dichroic ratio about 1.6; the experimental value for the peak absorbance with unpolarized radiation is not more than 3% lower than the calculated figure.

In a critical evaluation of quantitative cytochemical techniques Glick, Engström, and Malmström[136] accept the conclusions of Thorell and Ruch, and cite other examples of biological material in which orientation might be expected but where the observed ultraviolet dichroism is low, so that absorption measurements with unpolarized radiation should not be subject to appreciable error. They also draw attention to the possibility of error due to inhomogeneous distribution of absorbing material over the total area of measurement.[137] It can easily be shown that error due to such inhomogeneity is most serious at high absorbance values. Since the proportional effect of a constant fractional error in transmission on absorbance (and hence on concentration) measurement also depends on the absorbance, Glick *et al.* emphasize the importance of working at fairly low absorbance levels. The concept of an optimum absorbance range, approximately 0.2–0.7, for precise spectrophotometry has long been accepted for measurements in solution, where problems due to heterogeneity do not arise.[138,139]

In a discussion of errors in microspectrography Wilkins[140] comments on the views of Commoner and Lipkin and concludes with Thorell and Ruch that errors due to orientation would only be serious in biological material of exceptional character, in which the degree of orientation is very high. In spite of considerable debate[141] it does not appear that Commoner and Lipkin's arguments have been completely refuted, however, and it seems likely that more experimental work on carefully chosen specimens will be necessary to resolve this issue.

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[136] D. Glick, A. Engström, and B. G. Malmström, *Science* **114,** 253 (1951).

[137] R. N. Jones, *J. Am. Chem. Soc.* **74,** 2681 (1952).

[138] G. F. Lothian, "Absorption Spectrophotometry," p. 52. Hilger and Watts, London, 1949.

[139] N. T. Gridgeman, *Anal. Chem.* **24,** 445 (1952).

[140] M. H. F. Wilkins, *Discussions Faraday Soc.* No. **9,** 363 (1950).

7. Dichroism of Stained Nucleic Acids

The combination of a dye with an oriented macromolecule may lead to orientation of the dye molecules, causing the visible light absorption of the dyed macromolecule to show dichroism. There is an extensive literature on the visible dichroism of substantively dyed cellulose fibers, and White and Elmes[142] have made some observations on dyed birefringent fibers of DNA and the corresponding nucleoproteins isolated from various human tissues. With pyronine and methyl green, the positive dye dichroism indicates that the absorbing groups of the dyes lie parallel to the fiber axis, although imperfectly oriented. For toluidine blue–stained fibers the visible dichroism is negative, and the absorbing groups of the dye appear to lie roughly at right angles to the fiber length. It is of interest to note that the oriented DNA fibers used in this work were laid down within crystals of sodium chloride depositing from solutions of the nucleic acid or nucleoprotein in sodium chloride solutions. The salt could be dissolved away with methanol leaving arrays of birefringent fibers which show the type A–type B interconversions described by Wilkins *et al.*[127] The dye dichroism of stained oriented nucleic acid is of great interest in relation to the use of methyl green as a specific dye for high-molecular-weight DNA; conversely pyronine stains depolymerized DNA and undegraded PNA. The differential staining of tissues by methyl green-pyronine (Unna-Pappenheim) is thus intimately related to the nature and state of the nucleic acids present.[143] The subject has been discussed in detail by Kurnick[144] and Thomas.[145] The phosphate groups are certainly implicated in the binding of basic dyes by nucleic acids,[146] but an explanation for the binding of specific dyes in terms of dye molecular structure has not yet been attempted. It is perhaps noteworthy that unlike other biological macromolecules with acidic functional groups, PNA is a unique substrate for some basic dyes in that it represses dimerization and hence the change in dye color known as metachromasy. The subject has been discussed by Michaelis.[147] [Cf. *Swift*, Chapter 17.]

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[141] B. Commoner, *Discussions Faraday Soc.* No. **9,** 393 (1950); H. R. Catchpole and I. Gersh, *ibid.*, p. 471.

[142] J. C. White and P. C. Elmes, *Nature* **169,** 151 (1952).

[143] J. Brachet, *Compt. rend. soc. biol.* **133,** 88 (1940).

[144] N. B. Kurnick, *Exptl. Cell Research* **1,** 151 (1950); *ibid.* **3,** 649 (1952); *Stain Technol.* **27,** 233 (1952); *Arch. Biochem.* **29,** 41 (1950).

[145] R. Thomas, *Arch. intern. physiol.* **61,** 270 (1953).

[146] L. F. Cavalieri and A. Angelos, *J. Am. Chem. Soc.* **72,** 4686 (1950); L. F. Cavalieri, S. E. Kerr, and A. Angelos, *ibid.* **73,** 2567 (1951).

V. Infrared Absorption Spectra

The application of infrared absorption techniques to the study of nucleic acids is relatively new as in Loofbourow's extensive review article published in 1940,[51] on the investigation of materials of biological interest by physical methods, there is not a single reference to this topic. Even Schlenk's account of nucleic acid chemistry which appeared in 1949[148] cites only one paper, that of Blout and Fields[149] published in 1948. Subsequent investigations on nucleic acids proper have been reviewed very recently by Fraser.[150] Several extensive studies, however, of simple pyrimidines and related compounds have been made and are briefly noted here.

Broadly speaking, infrared absorption methods have been used for two different purposes in the nucleic acid field. On the one hand, the wealth of detail in an infrared spectrum may be used as a "finger-print" to identify a particular compound, and to detect it in mixtures. The infrared absorption spectrum may also be used as a proof of identity between synthetic and natural specimens of the same compound, especially when the existence of isomers with very similar properties results in other characterization methods being inadequate for such a purpose. Many examples of this application can be found in the recent literature dealing with the synthesis of nucleosides and nucleotides. For example, Brown and Todd[151] were able to distinguish between natural and synthetic adenylic acids *a* and *b* and muscle adenylic acid (adenosine-5′-phosphate) by their infrared absorption spectra, obtained on mulled samples, and the isomeric cytidylic acids can also be identified in spite of the additional complication of polymorphism.[152] A strong characteristic band free from overlapping can be used for the estimation of a compound in mixtures if suitable solvents are available, though even mull spectra are of analytical value.[152] Work of this sort entails the use of infrared absorption spectra on an essentially empirical basis and no fundamental interpretation of the spectrum is really required. The other application is for structural studies, and in this case very detailed analysis of the spectrum is necessary. This may take the form of the correlation of band frequencies with particular structural elements of the sample, i.e., the use of "group correlations," for which there is an extensive empirical and theoretical literature (see Fraser[150] for references). For more fundamental indications of structure, e.g., the detection of hydrogen-bonding in polynucleotide chains and other detailed aspects of macromolecular structure, reliable assignments of bands to known modes of vibration are required. The progress which has been made in this direction has also been critically discussed by Fraser[150] for biological macromolecules, and by Thompson *et al.*[153] and Sutherland[154] for large molecules in general.

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[147] L. Michaelis, Nucleic acids and nucleoproteins, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 131–142 (1947); see also G. Oster and H. Grimsson, ref. 60.

[148] F. Schlenk, Chemistry and enzymology of nucleic acids, *Advances in Enzymol.* **9,** 455–535 (1949).

[149] E. R. Blout and M. Fields, *Science* **107,** 252 (1948).

[150] R. D. B. Fraser, The infra-red spectra of biologically important molecules, *Progr. Biophys. and Biophys. Chem.* **3,** 47–60 (1953).

[151] D. M. Brown and A. R. Todd, *J. Chem. Soc.* **1951,** 44.

[152] R. J. C. Harris, S. F. D. Orr, E. M. F. Roe, and J. F. Thomas, *J. Chem. Soc.* **1953,** 489.

Problems of sample preparation and of solvent selection are prominent in the infrared spectroscopy of nucleic acids and the related simple compounds. Because of its heavy absorption water is virtually useless as a solvent, quite apart from its solvent power for the rock-salt windows normally used in absorption cells. The available solvents with useful transmission windows in the infrared[155] are of little value; thus Lacher and coworkers[156] went to the trouble of using antimony trichloride at 100° C. to obtain absorption data in the 1–2.5-μ overtone region on some pyrimidines. Pyrimidines and purines have therefore mainly been examined as mulls, in which a finely powdered solid is mixed with a suitable nonvolatile liquid. The spectrum of a mulled sample will be partly obscured by any absorption bands of the mulling liquid, and the observed band intensities are subject to uncertainties arising from scattering losses and the difficulties in controlling sample concentration and film thickness. The fact that the spectrum refers to the solid state must also be borne in mind when theoretical interpretation is attempted. Many pyrimidines and purines are sufficiently stable to be sublimed *in vacuo*, however, and thin-film samples prepared in this way have been used by many workers for both infrared[149,157,158] and ultraviolet[33,34] absorption studies. A new method[159] for preparing solid samples for infrared absorption studies makes use of the fact that powdered potassium bromide can be converted under moderate pressure into clear plates with good infrared transmission. The finely ground sample is uniformly mixed with the halide salt before pressing, and its volume concentration in the resulting "sample plate" can be readily controlled. The spectra obtained from samples prepared in this way are free from solvent absorption and scattering errors and are of quantitative value, so that this technique promises to be a very real advance in infrared analytical technique.

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[153] H. W. Thompson, D. Nicholson, L. N. Short, *Discussions Faraday Soc.* No. **9,** 222 (1950).

[154] G. B. B. M. Sutherland, *Discussions Faraday Soc.* No. **9,** 274 (1950).

[155] P. Torkington and H. W. Thompson, *Trans. Faraday Soc.* **41,** 184 (1945).

[156] J. R. Lacher, D. E. Campion, and J. D. Park, *Science* **110,** 300 (1949).

[157] E. R. Blout and M. Fields, *J. Biol. Chem.* **178,** 335 (1949).

[158] E. R. Blout and M. Fields, *J. Am. Chem. Soc.* **72,** 479 (1950).

[159] M. M. Stimson, *J. Am. Chem. Soc.* **74,** 1805 (1952); U. Schiedt, *Z. Naturforsch* **76,** 270 (1952); *Appl. Spectroscopy* **7,** 75 (1953).

Nucleic acid samples can be examined as cast films, prepared by evaporating aqueous solutions on silver chloride plates.[149] Oriented samples can be obtained by shearing viscous gels or by stretching fibers;[103] such samples are usually too small to be examined directly with conventional spectrometers and require some form of reflecting microscope to form an enlarged image of the specimen on the entrance slit.[160]

The infrared absorption spectra of the pyrimidines and purines found in nucleic acids and of xanthine, hypoxanthine, and of some methylated xanthines have been measured by Blout and Fields,[149,157,158] mainly on evaporated films. Several features in the spectra that are of value for identification and analysis are noted and some bond assignments proposed. Brownlie[161] has examined twenty-five pyrimidines, containing two or more substituents, as mulls over the 2–15-μ range and has attempted a large number of possible bond assignments. Some of his conclusions are debated by Short and Thompson[162] in a study of no less than eighty pyrimidines including many important mono-substituted derivatives, over the range 2–25 μ. These workers used lithium fluoride, sodium chloride, and potassium bromide prisms to cover this wide range of wavelengths, and treated some of their compounds with deuterium oxide to assist the recognition of vibrations involving hydrogen atoms. Their discussion of this large volume of data is an excellent example of the partly empirical, partly theoretical approach that has to be employed in the interpretation of such complex spectra. All the spectra are highly characteristic and contain many strong sharp bands suitable for identification and analysis, even when precise structural correlations are difficult. Interpretations based on previously established group correlations favor a ketonic structure for the 2-hydroxy- and 4-hydroxypyrimidines and probably a diketonic structure for the 2,4-dihydroxy derivatives. Amino groups appear to exist in the non-tautomerized form. Both the tautomerism and the hydrogen-bonding in the solid state are influenced by the various substituents present in the compounds studied. Short and Thompson conclude that frequency assignments in pyrimidine spectra must be made with considerable reserve, in view of the meager X-ray structure data that can be utilized to provide confirmation. Even so, their tentative findings are obviously of great interest in

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[160] R. D. B. Fraser, *Discussions Faraday Soc.* No. **9,** 378 (1950).

[161] I. A. Brownlie, *J. Chem. Soc.* **1950,** 3062.

[162] L. N. Short and H. W. Thompson, *J. Chem. Soc.* **1952,** 168.

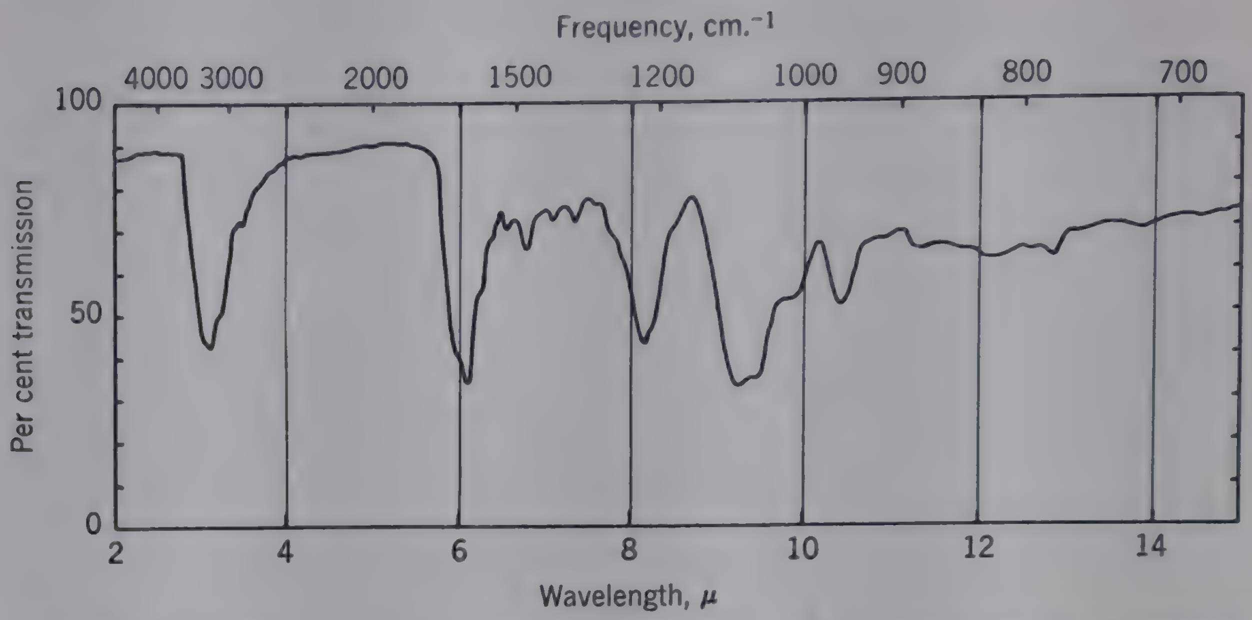


FIG. 28. Infrared absorption spectrum of cast film of DNA sodium salt (Rowen[163]).

relation to the specific interactions between base substituent groups envisaged in the Watson and Crick structure for DNA.[77]

With regard to infrared absorption studies on nucleic acids proper there is as yet very little to add to Fraser's excellent account,[150] which deals also with proteins and polysaccharides. The following frequency ranges are listed by him for the fundamental vibrations that may be readily recognized in the spectra of nucleic acids (e.g., Fig. 28).

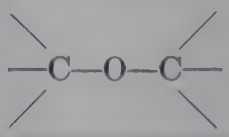
(1) Vibrations involving hydrogen atoms:

Bond stretching	O—H	3000–3700	cm.$^{-1}$
	N—H	3000–3500	"
	C—H	2800–3100	"
Bond bending	O—H	*ca.* 1100	"
	N—H	1500–1600	"
	C—H	1300–1500	"

(2) Multiple-bond stretching:

C=O	1600–1800	cm.$^{-1}$
C=N	*ca.* 1650	"
C=C	*ca.* 1650	"
P=O	1250–1300	"

(3) Skeletal frequencies involving many linked atoms:



$-C-O-C-$ *ca.* 1100 cm.$^{-1}$

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[163] J. W. Rowen, *Biochim. et Biophys. Acta* **10,** 391 (1953).

(4) Ionic group frequencies:

PO_4^{---} *ca.* 1080 and 980 cm.$^{-1}$

The large number of different atomic groupings in a biological macromolecule such as nucleic acid gives rise to a very complex set of characteristic and skeletal frequencies so that only a few of the more important can be assigned with any certainty. The situation is complicated by overlapping of bands and by severe broadening of those bands arising from groups which may be hydrogen-bonded. The most detailed analyses have been made on the spectra of proteins and related compounds,[164] and the interpretation of nucleic acid spectra has followed along essentially similar lines, extended to include the structural features peculiar to these compounds.

Blout and Fields[149,157] find that the sodium salts of PNA and DNA can be distinguished by their infrared absorption spectra, especially at frequencies lower than *ca.* 1100cm.$^{-1}$. Fraser and Chayen have used microspectrographic methods[165] to extend this finding to intact tissue sections. The specific frequencies recognized by these workers are 860, 916, 969, and 997 cm.$^{-1}$ for PNA, and 895, 930, and 967 cm.$^{-1}$ for DNA, as confirmed by selective extraction procedures. Since the technique can be made at least semiquantitative, it offers the possibility that the two types of nucleic acid can be differentiated and estimated separately *in situ* in biological material. A further advantage is that any mononucleotide absorption contributions can be distinguished and evaluated separately. Quantitative infrared microspectrographic procedures may therefore prove to be a valuable supplement to the more refined ultraviolet methods which have been given so much more attention in the past.

Macroscopic tissue sections have been studied by Blout and Mellors,[166] the samples being evenly wetted with high-molecular-weight fluorocarbon or hydrocarbon (mineral) oil to reduce scattering. These authors were particularly interested in the possibility of clinical diagnostic applications, and observed that a band of 9.3 μ (1075 cm.$^{-1}$), which they associated with nucleic acid, was more prominent in cancerous than in healthy mammary tissue. They also found definite differences in the spectra of fixed and unfixed samples of the same tissue.

Cavalieri, Kerr, and Angelos,[146] in a study of the enzyme-resistant residues of various PNA samples, concluded that the infrared absorption spectra of mulls were not sufficiently characteristic to reveal differences in macromolecular structure, although intact nucleic acids could be differentiated from mixtures of mononucleotides. More recently, however, Rowen[163] has examined the effect of treatment with deoxyribonuclease on the infrared absorption spectra of cast films of DNA (Fig. 28). The band frequencies and assignments proposed by this author are in substantial agreement with those given by Fraser;[150] the 960-cm.$^{-1}$ band is greatly reduced in intensity by enzymic degradation and is therefore correlated with the internucleotide ester linkage (P—O—C_5'), the 1015-cm.$^{-1}$ band then being regarded as arising in the nucleoside-phosphate bond (P—O—C_3'). The assignments of vibrations involving the phosphate group are very important for the interpretation of the infrared absorption and dichroism of nucleic acids and are briefly discussed by Fraser.[150]

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[164] G. B. B. M. Sutherland, Infrared analysis of the structure of amino acids, polypeptides and proteins, *Advances in Protein Chem.* **7,** 291–318 (1952).

[165] R. D. B. Fraser and J. Chayen, *Exptl. Cell Research* **3,** 492 (1952).

[166] E. R. Blout and R. C. Mellors, *Science* **110,** 137 (1949).

The dichroism of infrared absorption bands has been used in recent structural studies on nucleic acids. The theoretical basis of the method is the assumption that the transition moment associated with a vibration which is active in absorption is either along or perpendicular to the band involved, depending on whether the vibration is bond-stretching or bond-bending, respectively. The dichroism of the C═O stretching and N—H bending bands has been widely used to investigate the α- and β-configurations of oriented structural proteins and synthetic polypeptides (for literature references see Fraser[150] and Short and Thompson[162]). Fraser and Price[167] have shown, however, that in the important case of the peptide linkage this simple assumption may not be justifiable. A consideration of the mechanical interactions involving atoms other than the bonded pair in the C═O group, and of the effect of the vibration on the resonance structures of the peptide group, indicate that the direction of the transition moment for the C═O stretching vibration will not be exactly along the bond. The interpretation of infrared dichroism may therefore have to be made with considerable caution.

Fraser and Fraser[168] have studied the infrared absorption and dichroism of shear-oriented films of thymus DNA and assigned the principal bands in accordance with the general classification given above. The N—H stretching vibrations of the base amino and imino-groups and the double-bond (C═O, C═N, C═C) stretching vibrations of the same ring systems show perpendicular dichroism, i.e., absorption is greatest when the electric vector is perpendicular to the direction of shear, which is taken as the direction of the polynucleotide chain. In agreement with the negative birefringence and ultraviolet dichroism, the perpendicular infrared dichroism indicates that the planar bases are approximately perpendicular to the chain axis. The dichroism of other bands which are associated with the sugar residue and the phosphate group also appears to be consistent with the modifications proposed by Furberg[169] to the Astbury polynucleotide model. [Cf. *Jordan*, Chapter 13.] Fraser and Fraser have also found that the type A → type B transformation of DNA which occurs on stretching at a suitable humidity (cf. Section IV) is accompanied by a reversal from perpendicular to parallel of the dichroism of many important bands; the effect is most marked with the 967-cm.$^{-1}$ bond, which has not yet been assigned with certainty. The bond at 1235 cm.$^{-1}$ with a dichroic ratio of unity, is not affected by this structural transformation. The assignment of this bond to a P═O stretching vibration[167] has been reconsidered.[150]

Fraser[170] has studied the infrared dichroism of shear-oriented tobacco mosaic virus gels and finds the frequencies and dichroism of the C═O stretching and N—H bending vibration bonds to be consistent with a structure in which the protein is in an α-configuration with the polypeptide chains perpendicular to the axis of the rod-like virus particle, unlike some fibrous proteins which have been studied by the same method.

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[167] R. D. B. Fraser and W. C. Price, *Nature* **170,** 490 (1952); see also A. Elliott, *ibid.* **172,** 359 (1953).

[168] M. J. Fraser and R. D. B. Fraser, *Nature* **167,** 761 (1951).

[169] S. Furberg, *Acta Chem. Scand.* **4,** 751 (1950); see D. O. Jordan, ref. 6, for summary.

[170] R. D. B. Fraser, *Nature* **170,** 490 (1952).

Ambrose and Butler[171] have determined the dichroism in the 1500–1800-cm.$^{-1}$ region of herring sperm nucleoprotein oriented by being cast on a rubber film base which may be stretched to produce about 100% extension. The dichroism of the 1695-cm.$^{-1}$ stretching vibrations of the base C═O and C═N bonds are consistent with the dichroism found by Fraser and Fraser[168] for the moderately extended and optically negative type A nucleic acid fiber. The protein, however, is in the β-configuration in this nucleoprotein. There seem to be interactions between the two components of a nucleoprotein system which determine, on the one hand, the configuration assured by the protein and, on the other hand, the internal order of the polynucleotide chain.

It can be seen from this brief account of previous work that there are many practical and theoretical difficulties associated with the infrared absorption spectroscopy of nucleic acids. Further progress in the assignment of frequencies and in resolving uncertainties in the directions of transition moments should lead to significant contributions to knowledge of the detailed structure of nucleic acids.

Addendum

Bases, Nucleosides, and Nucleotides

Fox *et al.*[171a] report that the ultraviolet absorption spectra of cytidylic acids *a* and *b* behave in a significantly different manner in the alkaline pH range, and attribute this to the presence or absence of an ionizable 2′-hydroxyl group. The spectrum of cytidylic acid *a*, like that of 2′-deoxycytidine, remains constant in the pH 12 to 14 region, and hence may be presumed to be the 2′-phosphate ester, in which no 2′-hydroxyl group ionization can occur. This agrees with other evidence. The specific effect of 2′-hydroxyl ionization in the *b* or 3′-isomer may be due to hydrogen bonding between this group and the 2-keto group of the pyrimidine nucleus, since in the 2′- and 3′-adenylic acids, where such hydrogen-bonding cannot occur, no changes in the alkaline pH region are observed with either isomer. Spectroscopic constants for the isomeric cytidylic acids have also been reported by Harris *et al.*[152] and by Cavalieri[171b].

E. L. Bennett[172] and L. L. Bennett[173] have synthesized a number of C^{14}-containing purines and pyrimidines, respectively, and report brief spectroscopic data which are in good agreement with values for the normal compounds. Cavalieri *et al.*[174] have discussed the effects of methyl substitution on the ultraviolet absorption spectra of xanthines and give absorption curves for xanthine and xanthosine which agree well with those published here. Hamer *et al.*[175] have reported the effects on the ultraviolet absorption spectra of purines and pyrimidines when these compounds are reduced under acid and alkaline conditions.

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[171] E. J. Ambrose and J. A. V. Butler, The physical chemistry of proteins, *Discussions Faraday Soc.* No. **13,** 261 (1953).

[171a] J. J. Fox, L. F. Cavalieri, and N. Chang, *J. Am. Chem. Soc.*, **75,** 4315 (1953).

[171b] L. F. Cavalieri, *J. Am. Chem. Soc.*, **75,** 5268 (1953).

[172] E. L. Bennett, *J. Am. Chem. Soc.* **74,** 2420 (1952).

[173] L. L. Bennett, *J. Am. Chem. Soc.* **74,** 2432 (1952).

Nucleic Acids and Polynucleotides.

Laland *et al.*[175a] have determined ε(P) values for DNA preparations from animal, plant and microbial sources, before and after degradation by acid, alkali, heat, ultrasonic irradiation or desoxyribonuclease. From their results they conclude that any treatment of DNA which only leads to a decrease in intermolecular bonding does not alter the ε(P) value, whereas the breaking of intramolecular hydrogen bonds results in an increase in ε(P). These workers therefore prefer to regard any alteration in the state of DNA in solution which is not accompanied by an increase in ε(P) as a disaggregation, to distinguish it from the breakdown of the intermolecularly-bonded structure which gives rise to the absorption anomaly.

Ultraviolet Dichroism

Franklin and Gosling's X-ray diffraction studies of oriented DNA fibers have now been reported in greater detail.[176] These authors recognize a highly crystalline A-form, stable at 75% relative humidity, and a paracrystalline B-form which occurs at humidities of 92% and higher. Riley and Oster[177] had previously studied the DNA–water system over a very wide range of composition, and the micelle state in which DNA shows a partly ordered liquid–crystalline structure, may correspond to the paracrystalline B-form of Franklin and Gosling. The relation between the various forms of hydrated DNA recognized by X-ray diffraction and those recognized mainly by dichroism by Wilkins, Gosling, and Seeds[127] (Section IV.5) is not yet clear, but it seems certain that the macromolecular order, and presumably the optical properties of the oriented DNA–water system, are critically dependent on water content, as explicitly stated by Franklin and Gosling.

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[174] L. F. Cavalieri, J. J. Fox, A. Stone, and N. Chang, *J. Am. Chem. Soc.* **76,** 1119 (1954).

[175] D. Hamer, Deirdre M. Waldron, and D. L. Woodhouse, *Arch. Biochem. and Biophys.* **47,** 272 (1953).

[175a] S. G. Laland, W. A. Lee, W. G. Overend and A. R. Peacocke, *Biochim. et Biophys. Acta,* **14,** 356 (1954).

[176] R. E. Franklin and R. G. Gosling, *Acta Cryst.* **6,** 673, 678 (1953).

[177] D. P. Riley and G. Oster, *Biochim. et Biophys. Acta* **7,** 526 (1951).

Infrared Absorption Spectra

Blout and Lenormant[178] have obtained infrared absorption spectra of concentrated solutions of RNA, DNA, and DNP in both water and deuterium oxide, using very thin cells to reduce the solvent absorption. Under these conditions the absorption bands are sharper than those given by dried films. The characteristic 1020-cm.$^{-1}$ band of DNA is retained in solution with a slight shift in wavelength, and DNA and RNA can be clearly differentiated.

Frick and Rosenberg[179] have obtained some infrared absorption evidence for the existence of hydrogen bonds in native highly polymeric DNA. Working with pressed KBr sample plates[159] and a calcium fluoride prism, they find that a small maximum at *ca.* 3.1 μ, ascribed to hydrogen-bonded NH stretching, is reduced to an inflection by prior exposure of the sample to pH 12, the remainder of the spectrum being unaltered.

[178] E. R. Blout and H. Lenormant, *J. Opt. Soc. Amer.* **43,** 1093 (1953).

[179] G. Frick and A. Rosenberg, *Biochim. et Biophys. Acta* **13,** 455 (1954).

CHAPTER 15

Nucleases and Enzymes Attacking Nucleic Acid Components

G. SCHMIDT

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I. Introduction

Owing to the large number of the several types of bonds between the component groups in each nucleic acid molecule, various sequences of reactions are theoretically possible for the catabolism and for the biosynthesis of the nucleic acids. As in other metabolic fields, several different specific enzymes exist in many instances for the cleavage or formation of a given bond in nucleic acids or their derivatives. The pathway of nucleic acid metabolism is thus not uniform. The biological significance of the many alternative pathways of nucleic acid metabolism is not known at present, but the highly specialized distribution and the high specificity of some of these enzymes strongly suggests the idea that, in these instances, the enzymic degradation products have specific biological functions.

One generalization regarding the intermediary metabolism of nucleic acids can be made, at least as a summary statement reflecting the present state of knowledge: the only bonds known to be susceptible to the action of enzymes in nucleic acids or polynucleotides are the phosphoric ester linkages. All known deaminases or enzymes involving the glycosidic bonds act only on mononucleotides or their cleavage products. The degradation of nucleic acid in the laboratory to substances such as Gulland's deaminoribonucleic acid or Chargaff's apurinic acid has so far no analogy in the living organism. Obviously, confirmation or refutation of this statement will be an important topic of future research. The validity of this generalization would mean that the polymerization of mononucleotides would be the exclusive mechanism of the biosynthesis of the nucleic acids and the only determining factor for the sequence of the various nucleotide groups. It would mean that actions such as the enzymic exchange of purine groups would take place exclusively at the mononucleotide or nucleoside level.

The degrees of specificity of the enzymes of nucleic acid metabolism show widely different ranges. Many of these enzymes catalyze reactions not only of natural substrates, but also of analogous substances. Such cases are of particular interest when biosynthetic enzyme reactions are involved. The observations concerning the incorporation of antimetabolites into important cell constituents of the intact organism are paralleled by the behavior of isolated enzyme fractions. The concept of such incorporation is becoming increasingly important in the explanation of physiological effects of certain antimetabolites.

The number of different enzymic mechanisms in the field of nucleic acid metabolism is too large to fit into the original system of nomenclature proposed by Levene and Medigreceanu.[1] The present terminology is somewhat arbitrary because some original designations are now applied with a modified meaning. Levene and Medigreceanu introduced the collective term "nucleases" for all enzymes involved in the metabolism of nucleic acids or their degradation products or precursors. In contrast to this broad application of the term "nucleases,"[2] the terms "ribonuclease" and "deoxyribonuclease"—introduced by Dubos and Thompson[3] and by Kunitz,[3a] respectively—are now usually limited to the designation of enzymes which catalyze the cleavage of the phosphoric ester bonds interlinking the nucleotide groups of nucleic acids or polynucleotides.[4]

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[1] P. A. Levene and F. Medigreceanu, *J. Biol. Chem.* **9,** 389 (1911).

[2] O. Hoffmann-Ostenhof, *Advances in Enzymol.* **14,** 219, 225 (1953).

[3] R. Dubos and R. H. S. Thompson, *J. Biol. Chem.*, **124,** 501 (1938).

[3a] M. Kunitz, *J. Gen. Physiol.*, **33,** 349 (1950).

[4] H. S. Loring and F. H. Carpenter suggested replacement of the term "ribonuclease" by the term "ribonucleinase" [*J. Biol. Chem.* **150,** 381 (1943)], but the customary use of the shorter term is continued in the literature. The reviewer feels that, owing to the overlapping specificities of many nucleases, it will be next to impossible to devise a framework of nomenclature which will not be riddled with inconsistencies by future observations.

Only a few of the known enzymes of nucleic acid metabolism have been thoroughly investigated, and, as yet, only two have been obtained in crystalline form. The properties of these nucleases will be discussed in some detail in the following sections. Some of these enzymes have become important tools for the study of the structure of nucleic acids. The wealth of information accumulated during the intense recent study of ribonuclease I and deoxyribonuclease I is in striking contrast to the scarcity of descriptive data available for the majority of the enzymes of nucleic acid metabolism. A review of the present knowledge of this field is, therefore, of necessity very unbalanced. It should be pointed out that the relative space devoted to some individual enzymes reflects the intensity of work devoted to their study, rather than the degree of their importance in nucleic acid metabolism.

II. Enzymes Catalyzing the Cleavage of Bonds Between Nucleotides

1. Ribonucleases

a. Pancreas Ribonuclease (Ribonuclease I)

History. In 1920, Walter Jones observed that boiled extracts of pancreas were capable of transforming yeast ribonucleic acid to acid-soluble products without the liberation of inorganic phosphate or of purine and pyrimidine bases.[5] This observation was a decisive advance in our knowledge of nucleic acid metabolism because it demonstrated for the first time the existence of a specific enzyme the activity of which is limited to the cleavage of internucleotide bonds without the formation of ammonia, inorganic phosphate, or free purines or pyrimidines. The separation of this specific activity from those of the rather ubiquitous deaminases, phosphatases, and nucleosidases was facilitated by the exceptional heat stability of Jones' enzyme, since at the time of its discovery the technique of enzyme fractionation was very primitive. The enzymic degradation of nucleic acids in pancreas had been observed much earlier by Jones himself[6] as well as by others; but, in these experiments, the hydrolysis always proceeded beyond the stage of nucleotides. It is, therefore, justified to consider the date of the discovery of ribonuclease as coincident with that of the detection of its heat stability. Interestingly enough, in his first paper, Jones himself expressed doubt as to whether the activity of his boiled pancreas extracts could be attributed to the presence of an enzyme. This doubt was shared by his contemporaries and was during a considerable period a discouraging influence on the further investigation of Jones' observation. Eighteen years had elapsed after the publication of Jones' paper, when interest in the heat-stable nuclease of pancreas was reawakened by the observation of Dubos and his associates,[3,7] who found that the Gram-positive staining properties of killed pneumococci disappeared after incubation of the cells with boiled pancreas extracts. They succeeded in a considerable purification of Jones' enzyme for which they proposed the term "ribonuclease," and they found evidence supporting the assumption that the ribonucleic acid fraction of pneumococci was related to their Gram-positive properties. The work of these authors stimulated P. A. Levene[8] to undertake a study of pancreas ribonuclease in collaboration with Schmidt. These authors confirmed Jones' concept that the action of ribonuclease was limited to the cleavage of the interlinkages between the nucleotide groups of ribonucleic acid, but they emphasized that only part of these interlinkages could be split by the enzyme.

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[5] W. Jones and M. E. Perkins, *Am. J. Physiol.* **55**, 557 (1923).

[6] W. Jones, *Z. physiol. Chem.* **41**, 101 (1904).

A new phase in the investigation of ribonucleases began in 1940 when Kunitz[9] obtained the enzyme in crystalline form (Fig. 1) and found that its action was accompanied by the liberation of acidic groups. It will be seen in the following sections and in other chapters of this book that this achievement not only opened the way for a clearer understanding of the properties of Jones' enzyme, but that it also created a valuable tool for the study of the structure of its substrates, the ribonucleic acids.

Specificity. Ribonuclease I is a highly specific phosphodiesterase which hydrolyzes all known ribonucleic acids, certain ribonucleotides,[10] and synthetic ribonucleotide-P-esters[11] which will be defined in the following sections (see also Chapter 12), and a natural polymer of ribosephosphoric acid which was discovered by Zamenhof *et al.*[12] (Fig. 2). It also hydrolyzes polynucleotides obtained by deamination of PNA with nitrous acid.[13,14] On the other hand, ribonuclease is entirely inactive toward deoxyribonucleic acid and its hydrolysis products,[9] except apurinic acid ("thymic acid"),[15] and toward the P-esters of α-glycerophosphoric acid such as L-α-glycerylphosphorylcholine and L-α-glycerylphosphorylethanolamine.[14] Diphenyl phosphate and dinitrophenyl phosphate, which are used as

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[7] R. H. S. Thompson and R. Dubos, *J. Biol. Chem.* **125,** 65 (1938).

[8] G. Schmidt and P. A. Levene, *J. Biol. Chem.* **126,** 423 (1938).

[9] M. Kunitz, *J. Gen. Physiol.* **24,** 15 (1940).

[10] D. M. Brown, D. I. McGrath, and A. R. Todd, *J. Chem. Soc.* **1952,** 2708.

[11] D. M. Brown, E. A. Dekker, and A. R. Todd, *J. Chem. Soc.* **1952,** 2715.

[12] S. Zamenhof, G. Leidy, P. L. FitzGerald, H. E. Alexander, and E. Chargaff, *J. Biol. Chem.* **203,** 695 (1953).

[13] W. E. Fletcher, J. M. Gulland, D. O. Jordan, and H. E. Dibben, *J. Chem. Soc.* **1944,** 30.

[14] L. Vandendriessche, *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **27,** 341 (1951).

[15] M. C. Durand and R. Thomas, *Biochim. et Biophys. Acta* **12,** 416 (1953).

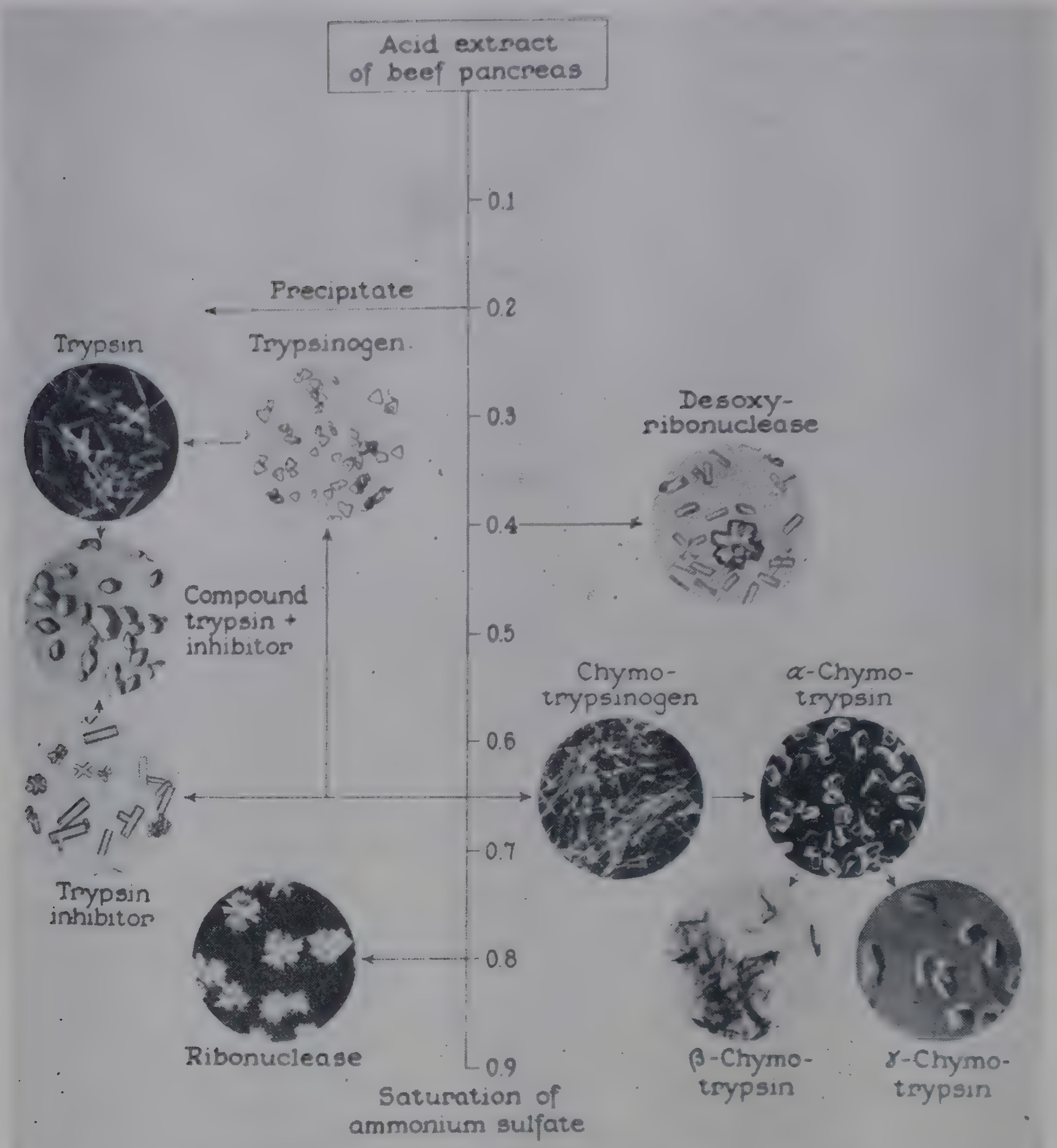

FIG. 1. Fractionation of pancreas enzymes. [From M. Kunitz.[3a]]

model substrates for other phosphodiesterases, are resistant against ribonuclease.[16]

Hydrolysis Products Resulting from the Action of Ribonuclease I on Ribonucleic Acids. Ribonuclease I does not hydrolyze all bonds between the component nucleotides of ribonucleic acid, but it catalyzes the cleavage only of certain strictly defined internucleotide bonds, namely those between the 3′-pyrimidine nucleoside phosphoryl groups and the 5′-hydroxy groups of the adjacent purine or pyrimidine nucleotide groups.[16,16a] Thus, neither the bonds between adjacent purine nucleotide groups nor

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[16] G. Schmidt, R. Cubiles, N. Zöllner, L. Hecht, N. Strickler, K. Seraidarian, M. Seraidarian, and S. J. Thannhauser, *J. Biol. Chem.* **192,** 715 (1951).

[16a] H. S. Loring, F. H. Carpenter, and P. M. Roll, *J. Biol. Chem.* **169,** 601 (1947).

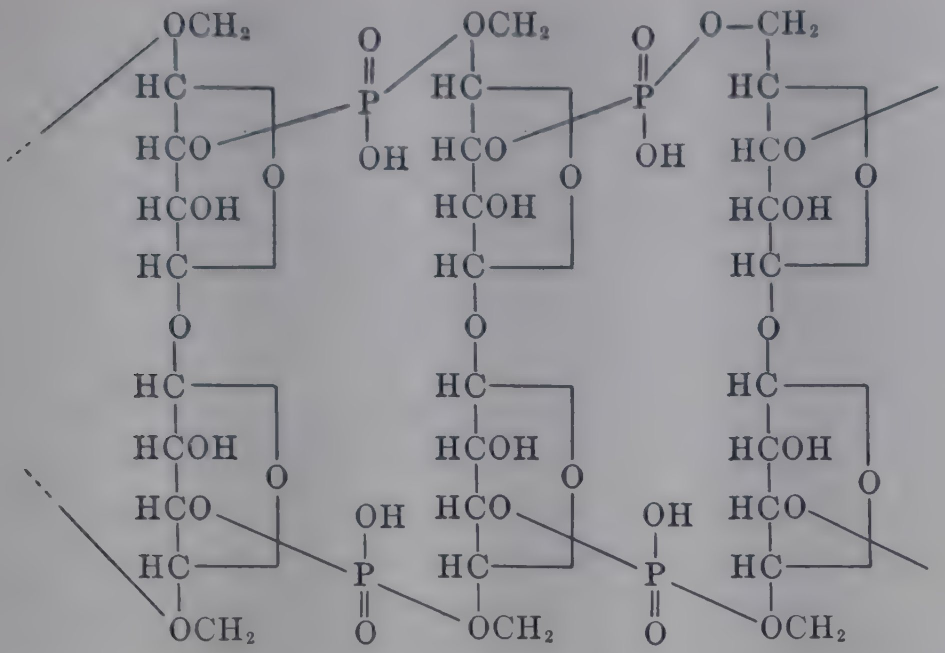

FIG. 2. Probable structure of ribose phosphate polysaccharide of *Hemophilus influenzae*. (From Zamenhof *et al.*[12])

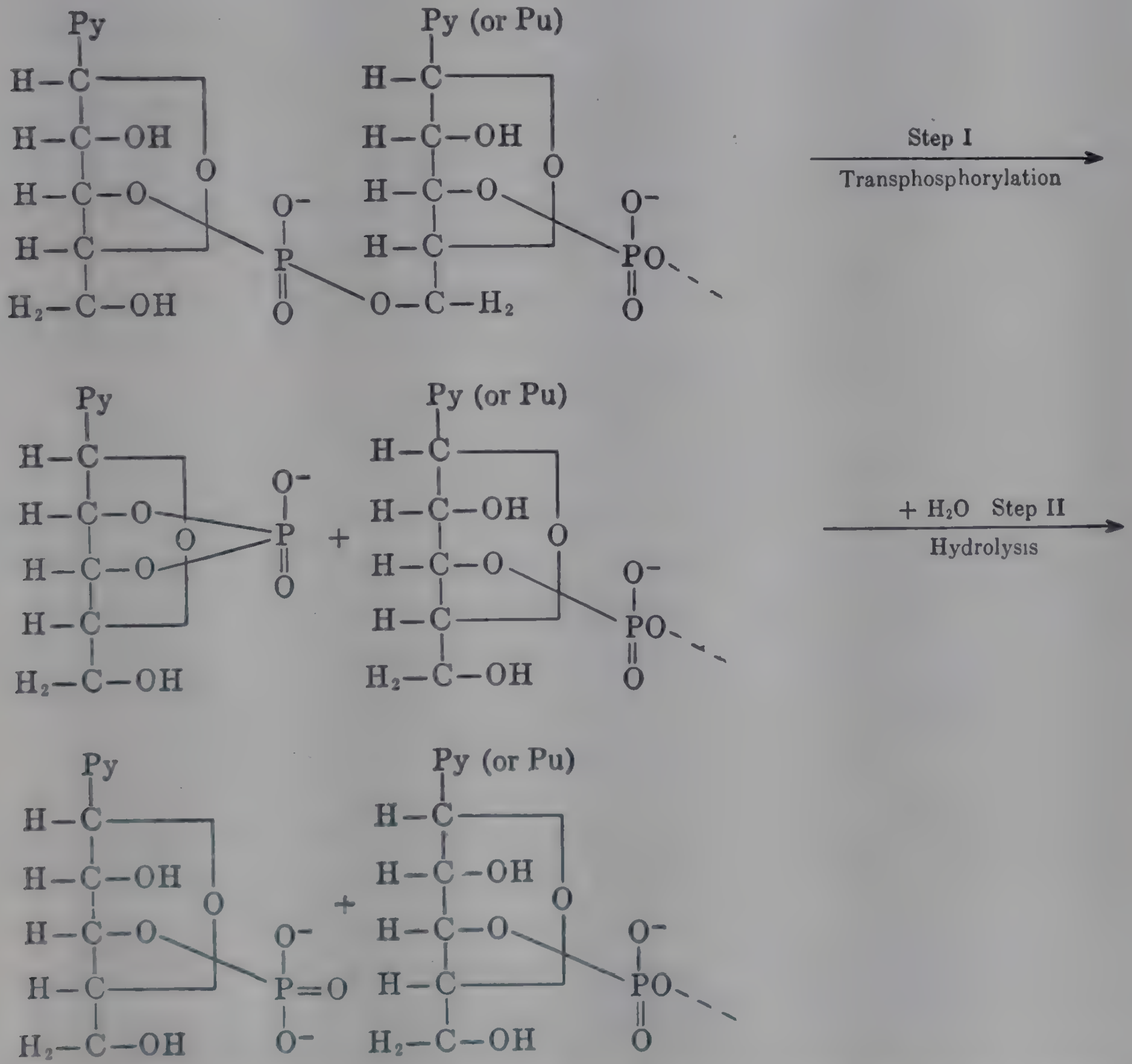

FIG. 3. Mechanism of action of ribonuclease I according to Brown and Todd.

the bonds between a 5′-pyrimidine phosphoryl group and a 3′-hydrox group of the adjacent purine or pyrimidine nucleotide group are hydrolyzed. Consequently, after exhaustive incubation with ribonuclease, al 3′-phosphoryl groups attached to pyrimidine nucleoside groups are presen in the form of secondary phosphoryls. This observation—in addition t kinetic observations—supports the conclusion that the incomplete hydrolysis of the internucleotide bonds by ribonuclease is not due to inhibitor influences on the enzyme, but to its sharply defined specificity toward th internucleotide bonds as defined in this chapter. The products obtaine after exhaustive digestion of ribonucleic acids with ribonuclease are 3′ uridylic acid, 3′-cytidylic acid, and a considerable number of oligonucleotides of various degrees of polymerization. These oligonucleotides wer designated as limit polynucleotides by Schmidt *et al.*[16] in analogy to th terminology used for the end-products of amylase action on polysaccharides. The simpler components of the complex mixture of the limit polynucleotides of low molecular weights were successfully separated by Volki and Cohn[17] on ion-exchange columns and by Markham and Smith[18] b ionophoresis. They are unbranched di-, tri-, and tetranucleotides of different compositions, but all have in common one structural property: eacl of these oligonucleotides contains one pyrimidine nucleotide group pe molecule. The pyrimidine nucleotide group is always terminal and carrie the secondary phosphoryl group of the chain on its 3′-carbon atom. Between 30 and 40% of the purines of yeast PNA and between 45 and 50% of those of liver PNA are found as oligonucleotides containing four o fewer mononucleotide groups after exhaustive digestion with ribonuclease The remainder are present in the form of polynucleotides of higher orde which have not been separated as yet.

A considerable proportion of these oligonucleotides, corresponding to 1 to 25% of the total phosphorus of the substrate, is not dialyzable against water. Until recently this fraction was considered to consist of polynucleotides of relatively high molecular weight which have been termed "cores' or "limit nucleic acids" by Zamenhof and Chargaff.[19] Markham anc Smith[18] showed recently, however, that the diffusibility of oligonucleotide through dialysis membranes was greatly enhanced by the presence o sufficient concentrations of sodium chloride in the solution.

The description of the internucleotide bonds hydrolyzed by ribonucleas implies that the degree of polymerization is not important for the actio of this enzyme.[20] This conclusion is borne out by the observations of Merrifield and Woolley,[21] who succeeded in isolating by ion-exchange chromatography a considerable number of di- and trinucleotides from hydrolysates of yeast ribonucleic acid obtained by short treatment of PNA with 6 N hydrochloric acid at room temperature. All those oligonucleotides in which the 3′-phosphoryl groups of pyrimidine nucleotides were esterified with other nucleotides were cleaved by ribonuclease, whereas all oligonucleotides in which such a structure was absent were resistant toward the enzyme.

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[17] E. Volkin and W. E. Cohn, *J. Biol. Chem.* **205,** 767 (1953).

[18] R. Markham and J. D. Smith, *Biochem. J.* **52,** 565 (1952).

[19] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **178,** 531 (1949).

[20] B. Magasanik and E. Chargaff, *Biochim. et Biophys. Acta* **7,** 396 (1951).

The presence of pyrimidine residues in those nucleotide groups which react with ribonuclease is, however, not a specific requirement for the catalytic action of the enzyme. Zamenhof *et al.*[12] isolated from *Hemophilus influenzae*, type b, a polymer of ribosyl-3-phosphoric acid which was hydrolyzed by ribonuclease (Fig. 2). It would appear, then, that the contrast in the behavior of pyrimidine and purine nucleotide groups against ribonuclease must be interpreted as a specific resistance of the purine nucleotide groups against the action of ribonuclease rather than by a specific importance of the pyrimidine group for the catalytic activity of this enzyme.

Mechanism of Action of Ribonuclease (see also Chapter 12). The enzymic cleavage of internucleotide bonds by ribonuclease is not a simple hydrolysis but an intramolecular transphosphorylation followed by hydrolysis. The presence of transphosphorylating and hydrolyzing activities in the same enzyme is not a unique property of ribonuclease, but has recently been observed in phosphomonoesterases. However, transphosphorylation as a necessary intermediary step of hydrolysis on the substrate level has so far been established only for the action of ribonuclease on polynucleotides.[22]

The concept of the biphasic nature of the cleavage of ribonucleic acids by ribonuclease is based on the following observations. Chantrenne, Linderstrøm-Lang, and Vandendriessche[23-25] made the interesting discovery that the volume changes of a ribonucleate solution during the action of ribonuclease were biphasic: A short and transitory dilatation of the solution was followed by a slow contraction. Since the simple hydrolytic cleavage of an ester bond would be expected to result exclusively in a small contraction, these authors concluded that the cleavage of the internucleotide bonds of ribonucleic acid by ribonuclease was not a simple hydrolysis, but that it followed a more complex mechanism.

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[21] R. B. Merrifield and D. W. Woolley, *J. Biol. Chem.* **197,** 521 (1952).

[22] As contrasted with hypothetical transphosphorylation between an "active" enzyme phosphate compound and the substrate.

[23] H. Chantrenne, K. Linderstrøm-Lang, and L. Vandendriessche, *Nature* **159,** 877 (1947).

[24] L. Vandendriessche, *Compt. rend. trav. lab. Carlsberg, Ser. chim.* **27,** 341 (1951).

[25] L. Vandendriessche, *Acta Chem. Scand.* **7,** 699 (1953).

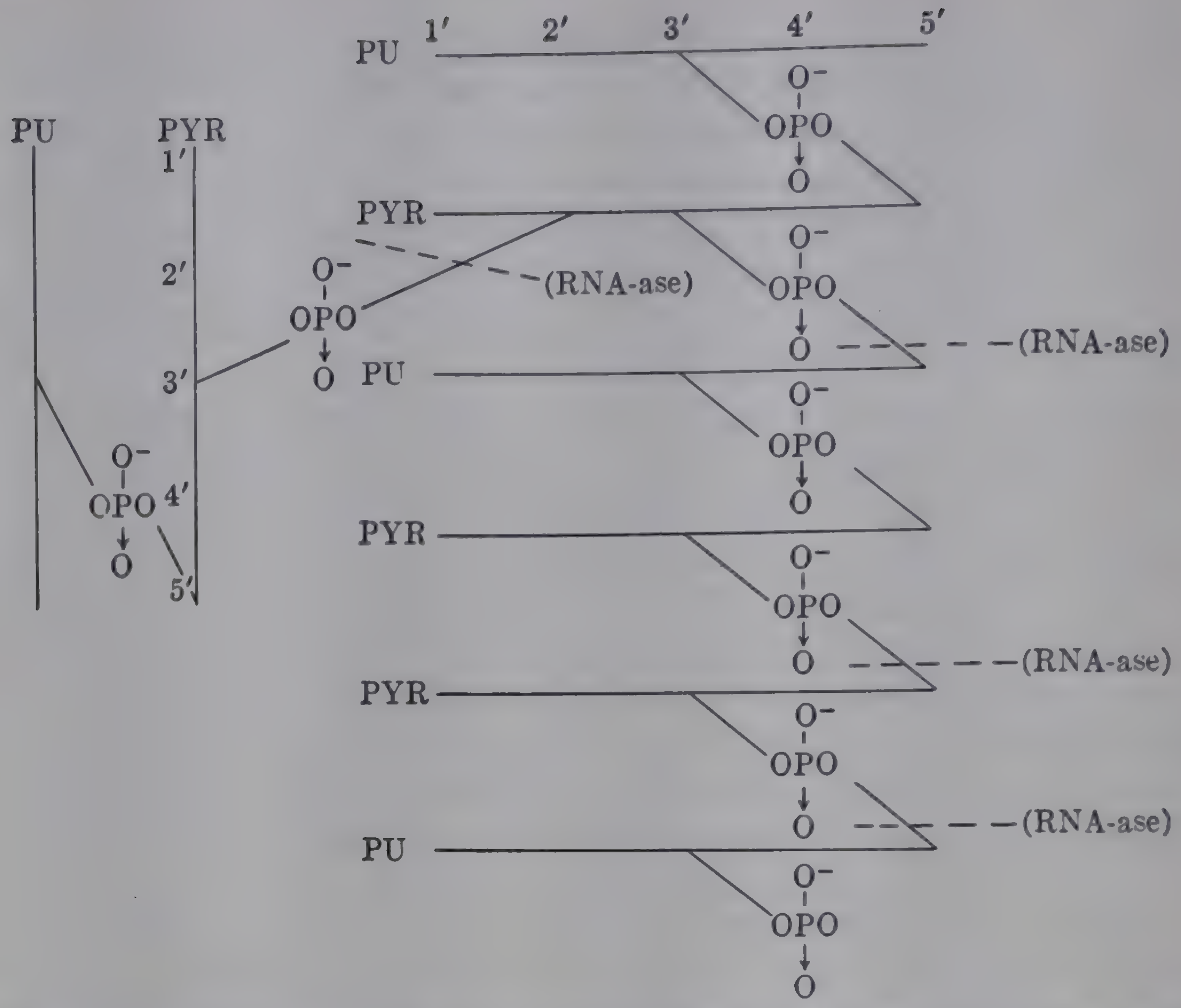

FIG. 4. Interlinkages in oligo- and polynucleotides and in ribonucleic acids cleaved by ribonuclease I. [Modified from the scheme of W. E. Cohn *et al. in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 344. Johns Hopkins Press, Baltimore, 1952.]

These physicochemical changes are explained by observations of Brown, Dekker, and Todd[26] and of Markham and Smith[27] that cyclic 2′,3′-nucleoside phosphoric acid diesters are intermediaries in the cleavage of the internucleotide bonds by ribonuclease.[28] In a second step the cyclic pyrimidine nucleotides are hydrolyzed by ribonuclease to ordinary 3′-nucleotides (or nucleotide groups). The diphasic enzymic cleavage of the internucleotide bonds is a peculiarity of the action of ribonuclease I on ribonucleic acid and ribopolynucleotides. These substrates have the structural requirements for such a reaction mechanism because they have 2′-hydroxy groups in the vicinal position to the corresponding 3′-phosphoryl groups of the internucleotide bonds (Fig. 4). The action of deoxyribonuclease on deoxyribonucleate causes only the usual volume contraction of 2 cm. per equivalent and is not accompanied by an intermediary phase of dilatation.

[26] D. M. Brown, C. A. Dekker, and A. R. Todd, *J. Chem. Soc.* **1952,** 512.

[27] R. Markham and J. D. Smith, *Biochem. J.* **52,** 552 (1952).

[28] L. A. Heppel, P. R. Whitfeld, and R. Markham, *Biochem. J.* **56,** Proc. iii (1954).

The intermediary formation of 2′,3′-cyclic pyrimidine nucleotides was suggested by the observation that they could be detected by paper ionophoresis of ribonuclease digests obtained by very short incubation of ribonucleate with very small quantities of the enzyme. They could be accumulated in considerable quantities when the digestion was carried out in dialysis bags so that the cyclic nucleotides were separated from the enzyme soon after they had been formed. The absence of cyclic nucleotides in later stages of the incubation or in digests obtained with large amounts of ribonuclease is explained by the observation that ribonuclease catalyzes the hydrolysis of cyclic pyrimidine nucleotides to the corresponding 3′-nucleotides. As shown in Fig. 3, it was concluded that the cleavage of the phosphoryl diester bonds by ribonuclease is not a simple hydrolysis, but a transphosphorylation (step I) followed by hydrolysis (step II). If A is a pyrimidine nucleotide with a 3′-phosphoryl group, and B the adjacent nucleotide esterified with this phosphoryl group at the 5′-position, the first rapid step of ribonuclease action consists in the shift of the 5′-phosphoryl ester bond of B to the 2′-position of A with the formation of a cyclic diester of phosphoric acid (cyclic nucleotide group or cyclic 3′-nucleotide). The second slow step in ribonuclease action is the specific enzymic hydrolysis of the cyclic diester at the 2′-phosphoryl ester linkage with the formation of a 3′-nucleotide group (or nucleotide).

Nucleotide-P-esters as Substrates of Ribonuclease. Brown and Todd succeeded in synthesizing model esters of nucleotides with benzyl, methyl and ethyl alcohols. It was found that the esters of 3′-cytidine and 3′-uridine phosphates were split by ribonuclease, but that those of the 5′-nucleotides and all esters of adenine nucleotides were resistant to the enzyme. The intermediary formation of cyclic nucleotides was demonstrated during the cleavage of all synthetic nucleotide esters susceptible to the action of the enzyme.[26]

The behavior of synthetic nucleotide-P-esters of known structure is thus in complete analogy to that of polynucleotides and shows that only one of the two ester bonds of phosphoric acid must be attached to a pyrimidine nucleoside to make the compound susceptible to the action of ribonuclease, and that this bond must be attached to the 3′-hydroxy group of the ribose moiety.

Some Synthetic Reactions Catalyzed by Ribonuclease. The conversion of internucleotide bonds to cyclic phosphodiester bonds by ribonuclease raises the question as to whether or not the reverse reaction might occur between cyclic nucleotides and other hydroxy compounds under the influence of this enzyme. Whereas the former reaction results in degradation, the latter would lead to the formation of larger molecules. Model reactions of such a synthetic action of ribonuclease have been demonstrated by

Heppel and Whitfeld,[29,30] who found that ribonuclease catalyzes the enzymic synthesis of benzyl- and of methylcytidylic acid from cyclic 2′,3′-cytidylic acid and the corresponding alcohols (which must be present in excess). Similarly, the incubation of a mixture of cyclic 2′,3′-cytidylic acid with cytidine results in the formation of the nucleotide-nucleoside-P-diester cytidylyl-cytidine,[30] that of cyclic uridylic acid and cytidine in the formation of uridylyl-cytidine. The observed transesterification reactions catalyzed by ribonuclease are not limited to the cyclic nucleotides as substrates, but occur between acyclic P diesters of pyrimidine-3′-nucleotides and other alcohols: for example, the incubation of a mixture of P-methyl-3′-cytidylic acid with cytidine results in the formation of cytidylyl-cytidine and methyl alcohol. Finally, the formation of oligonucleotides by enzymic exchange reactions of cyclic nucleotides with pyrimidine-3′-nucleotides were reported by Heppel *et al.*[30a]

The possible occurrence of such exchange reactions must of course be carefully considered in the interpretation of experiments carried out with ribonuclease as a hydrolyzing agent in investigations concerning the structure of PNA.

Principles of Assay of Ribonuclease. (*a*) *Formation of acid-soluble degradation products of PNA.*[31,32] The mononucleotides and many oligonucleotides are soluble at pH values of 2, whereas PNA is precipitated under these conditions. Hydrochloric (0.5 *N*), sulfuric, perchloric acids or glacial acetic acid have been used for such partitions. The precipitation of undigested nucleic acid in easily filtrable form is facilitated by the use of alcohol-containing acid solutions.[33] The fine dispersion of nucleic acid precipitates obtained in very dilute substrate solutions with aqueous acids has been used as a principle for a turbidimetric assay method for ribonuclease.[34] The acid-soluble hydrolysis products can be determined in the filtrates by phosphorus determination or by ultraviolet spectrophotometry. Roth and Milstein[33] used P^{32}-labeled PNA as substrate and determined the radioactivity of the filtrates obtained after incubation with ribonuclease-containing tissue extracts.

A very suitable reagent for the assay of ribonuclease by determination of its acid-soluble degradation products is 1.5% uranyl chloride in 10% of trichloroacetic acid.[35] According to MacFadyen,[35] this reagent precipitates

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[29] L. A. Heppel and P. R. Whitfeld, *Biochem. J.*, **56,** Proc. ii (1954).

[30] L. A. Heppel, R. Markham, and R. J. Hilmoe, *Nature* **171,** 1151 (1953).

[30a] L. A. Heppel, P. R. Whitfeld, and R. Markham, *Abstr. 126th Meeting Am. Chem. Soc., New York* p. 52c (1954).

[31] C. E. Carter and J. P. Greenstein, *J. Natl. Cancer Inst.* **1,** 29 (1946).

[32] A. Cantero, R. Daoust, and G. de Lamirande, *Science*, **112,** 221 (1950).

[33] J. S. Roth and S. W. Milstein, *J. Biol. Chem.* **196,** 489 (1952).

[34] M. McCarty, *J. Exptl. Med.* **88,** 181 (1948).

[35] D. A. MacFadyen, *J. Biol. Chem.* **107,** 297 (1934).

PNA quantitatively in flocculent form even from very dilute solutions, but does not precipitate mononucleotides and oligonucleotides of low molecular weight. Care must be taken to have the uranyl chloride present in excess when concentrated solutions of PNA are used as substrate. The acid-soluble fraction contains the mononucleotides and probably the dinucleotides quantitatively; some oligonucleotides of higher molecular weight, however, are difficultly soluble in acids and are partially precipitated together with PNA.

(*b*) *Formation of titratable acidic groups.* The cleavage of each nucleotide interlinkage involving a diesterified phosphoryl group results in the appearance of a titratable secondary phosphoryl group. The appearance of acidic groups during ribonuclease action was established by Kunitz[9] and by Allen and Eiler.[36] When ribonucleates are used as substrates, the accuracy of titration is limited by the possibility of pK shifts during their enzymic degradation. In addition, the interpretation of the titration curves is impeded by the overlapping of the dissociation ranges of the amino groups with the beginning, and of those of the phenolic groups with the end, of the dissociations of the secondary phosphoryls. According to Schmidt, Seraidarian, and Thannhauser,[37] this difficulty can be overcome by calculating the amounts of secondary phosphoryl groups from the slope of the titration curve between two close pH values in the neighborhood of pH 6 (e.g., between pH 5.9 and pH 6.2). In this range, which is the region of the pK_2 values of the nucleotides, the dissociation of the amino groups is negligible.

A simple transformation of the Henderson-Hasselbach equation shows that the amounts of secondary phosphoryl groups (T) in a nucleotide mixture can be calculated from the equivalents of alkali consumed between $(\mathrm{pH})_1$ and pH 8 (U_1), and those consumed between $(\mathrm{pH})_2$ and pH 8 (U_2) provided $(\mathrm{pH})_1$ and $(\mathrm{pH})_2$ are in the range between pH 5.8 and pH 6.4.

The equation is:

$$T = \frac{(U_1) \times \left[\dfrac{(\mathrm{H_2^+})}{(\mathrm{H_1^+})} - 1\right]}{\left[\dfrac{(\mathrm{H_2^+})}{(\mathrm{H_1})} \times \dfrac{U_1}{U_2}\right] - 1}$$

The validity of this equation can easily be checked with pure nucleotides for which the T values obtained for arbitrary pairs of $(\mathrm{pH})_1$ and $(\mathrm{pH})_2$ within the range from pH 5.8 and 6.4 are constant. For nucleic acids or nucleotide mixtures which contain different secondary phosphoryl groups with slightly varying pK values, constancy of the T values prevails only over a slightly narrower range of pH values, and the T values tend to decrease with the shift of the selected pair of (pH) values toward the alkaline range. It is easy, however, to find the range in which the decrease of T is minimal. The T value obtained in this range represents a close approximation to the correct value for the amount of secondary phosphoryl groups in the titrated sample.

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[36] F. W. Allen and J. J. Eiler, *J. Biol. Chem.* **137**, 757 (1941).

[37] M. Seraidarian, Thesis, Science Faculty, Tufts College, 1952.

These remarks will be sufficient to show that the increment of acidic groups during the hydrolysis of ribonucleic acid by ribonuclease can only be calculated from complete titration curves. This procedure is obviously too slow for measurements in the initial phases of the hydrolysis, but it is suitable for the determination of the final extent of hydrolysis. If only approximate measurements are required, the titrimetric technique can be adapted to determinations of hydrolysis by adjusting the pH of the substrate solution to a value around 8. The action of added ribonuclease results in a gradual decrease of the pH of the digest. At certain time intervals, the initial pH is reestablished by the addition of measured amounts of 0.1 N alkali. These amounts correspond to the newly formed acidic groups if pK shifts during the hydrolysis remain negligible, and if, in the pH range covered by the measurements, no titratable groups other than phosphoryl groups are liberated. The occurrence of pK shifts cannot be excluded, and the selection of pH 8 as end-point is arbitrary, particularly in view of the fact that the range of the inflection point of nucleotides in the region is usually very narrow and that the slopes of the titration curves of most oligonucleotides around these inflection points are rather steep.

Vandendriessche[24] as well as Cavalieri *et al.*[38] reported titrimetric observations from which they concluded that the action of ribonuclease results in the liberation of phenolic hydroxy groups the titration of which overlaps with that of secondary phosphoryl groups. The existence of internucleotide linkages involving phenolic hydroxy groups is by no means excluded by the wealth of recent evidence in favor of the concept that the majority of the internucleotide linkages are phosphoric acid ester bonds with 3′- and 5′-hydroxy groups, respectively.

(*c*) *Manometric determination of ribonuclease according to Bain and Rusch.*[39] The manometric determination of the liberated acidic groups offers the advantage that the initial stages of the hydrolysis can be quantitatively studied. Bain and Rusch reported a straight-line time–activity curve during the first 30 minutes of hydrolysis when sufficiently dilute enzyme solutions were used. Under such conditions, proportionality between the amounts of carbon dioxide developed and between the concentrations of ribonuclease were obtained. The procedure has been used for the determination of the concentration of ribonuclease in tissues.

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[38] L. F. Cavalieri, S. E. Kerr, and A. Angelos, *J. Am. Chem. Soc.* **73,** 2567 (1951).

[39] J. A. Bain and H. P. Rusch, *J. Biol. Chem.* **153,** 659 (1944).

The application of this method to kinetic studies, mainly by Zittle,[40] is complicated by the corrections which must be applied for the retention of carbon dioxide. The necessity for this correction interferes particularly with studies concerning Michaelis-Menten constants or concerning the effect of phosphate esters on the action of ribonuclease.

(*d*) *The secondary phosphoryl groups of ribonuclease digests* can also be determined enzymically with acid prostatic phosphatase as a specific hydrolyzing agent for secondary hydroxy groups. Schmidt *et al.*[16] found that monoesterified phosphoryl groups of nucleotides are rapidly and completely hydrolyzed by prostatic phosphatase, whereas diesterified phosphoryl groups are resistant toward this enzyme. These observations are in agreement with results obtained with chromatographic analyses of ribonuclease digests.[17,18,21] The transformation of phosphodiester groups into phosphomonoester groups by ribonuclease explains the fact that exhaustive digestion with prostate phosphatase releases a much larger amount of inorganic phosphate from ribonuclease digests than that formed under similar conditions from ribonucleic acid.[16,40,41]

So far, the phosphatase method has mainly been used for analyzing ribonuclease digests in the final phase of hydrolysis, whereas the others are better suited for enzyme assays or kinetic studies.

(*e*) *Light absorption in the ultraviolet region.* The action of ribonuclease on ribonucleic acids is accompanied by changes in the absorption of ultraviolet light for which no theoretical explanation can be given as yet. Kunitz[42] found a decrease of the absorption at 290 $m\mu$ which can be used for the assay of purified preparations of ribonuclease.

In the region around 260 $m\mu$, the characteristic range of the ultraviolet absorption of the bases, the action of ribonuclease causes no appreciable optical changes.[20,42] This is of interest since the quantitative degradation of PNA to mononucleotides by alkali is accompanied by an increase of approximately 20% in the absorption at 260 $m\mu$ (hyperchromic effect). The smaller absorption of PNA samples in comparison with the sum of the absorption effects of their mononucleotides is ascribed to an alteration of the resonance behavior of the bases when they are bound in polynucleotides of relatively high molecular weight. Magasanik and Chargaff[20] observed hyperchromic effects during the alkali hydrolysis of the high-molecular, but not of the low-molecular limit polynucleotides obtained by digestion of PNA with ribonuclease. They concluded from these observations that the structures responsible for the comparatively low ultraviolet absorption are mainly the purine nucleotide groups of the cores.

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[40] C. A. Zittle, *J. Biol. Chem.* **163,** 119 (1946).

[41] R. A. Bolomey and F. W. Allen, *J. Biol. Chem.* **144,** 113 (1942).

[42] M. Kunitz, *J. Biol. Chem.* **164,** 563 (1946).

Observations on the Kinetics of Ribonuclease. Substrates of well-defined and simple structure, i.e., oligonucleotides containing per molecule only one susceptible linkage or a well-defined number of such linkages would obviously be the most suitable materials for studies of the kinetics of ribonuclease as well as for its assay. Such substances, e.g., certain dinucleotides[21] or cyclic nucleotides,[10,11] became available only very recently, and no detailed kinetic studies on these highly interesting substrates have been published as yet.

For these reasons, all available kinetic data are based on the action of ribonuclease on ribonucleates. It is obvious that such data are of very limited value for the understanding of the action of ribonuclease. Many of the kinetic studies were carried out on commercial preparations of PNA. Recent experiences have shown that commercial preparations of yeast PNA must be considered as more or less degraded products which differ in many properties such as the number of terminal groups per molecule from PNA samples prepared by the mildest available procedures in the laboratory. But even genuine nucleic acid samples offer very complex conditions for kinetic studies since any ribonucleic acid preparation from a given tissue probably consists of a mixture of different nucleic acids and each individual PNA molecule contains a large number of linkages which are hydrolyzed by ribonuclease. Although some important features are common to all these linkages, they differ amongst each other in regard to their structure. It is very possible that the various linkages hydrolyzed by ribonuclease are cleaved at different rates so that the rate of ribonuclease action as measured, e.g., by the rate of formation of acidic groups from PNA, represents an overall rate resulting from a large number of individual enzyme reactions each of which might have its own characteristic kinetics.

According to the manometric measurements of J. A. Bain and H. P. Rusch,[39] the effect of the concentration of ribonucleate on the rate of ribonuclease action is very considerable; the maximal initial velocity is only approached at a substrate concentration of 6.5% (Fig. 5).

In addition, the curves of Figure 5 (according to Bain and Rusch[39]) demonstrate that relatively high substrate concentrations (at least 5%) are required in order to obtain constant hydrolysis rates during the initial phases of the enzyme reaction. At low substrate concentrations, the rates are falling continuously even during the first seconds of incubation.

This might suggest a strong competitive effect of the hydrolysis products of ribonuclease action on the enzyme. Actual data concerning such effects, however, are scanty and controversial. So far, only the effect of added mononucleotides has been studied and these experiments were carried out with mixtures of the 2′- and 3′-isomers.

Zittle[43] found approximately 50% inhibition in the presence of guanylic acid in 0.04 *M* concentration. Adenylic acid inhibited less than guanylic acid or than the mixture of the four nucleotides obtained by alkali hydrolysis from PNA. He defined the inhibition as noncompetitive, although the range of substrate concentrations studied appears as too narrow to permit this conclusion. He also found that commercial nucleic acid preparations (particularly free ribonucleic acid) contained acid-soluble contaminants which considerably inhibited ribonuclease action. This suggests inhibitory effects of acid soluble ribooligonucleotides. The available data must be considered as preliminary for reasons explained above. It should also be mentioned that the manometric determination of ribonuclease action, particularly in its initial phases, is not very accurate owing to the necessity to apply considerable and complicated corrections for the retention of carbon dioxide.

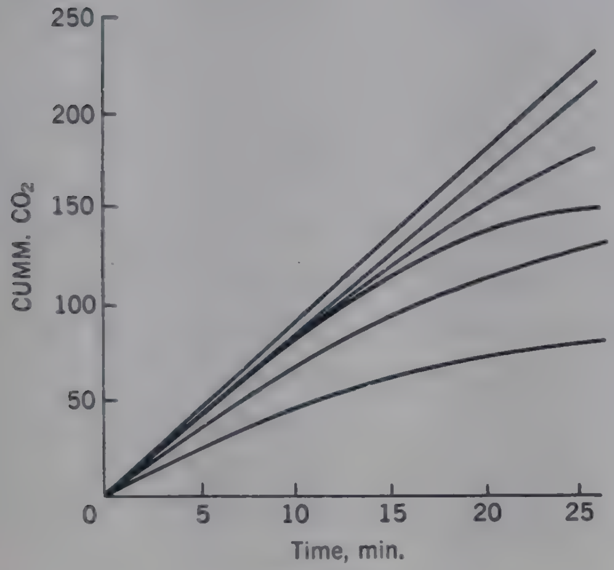

FIG. 5.

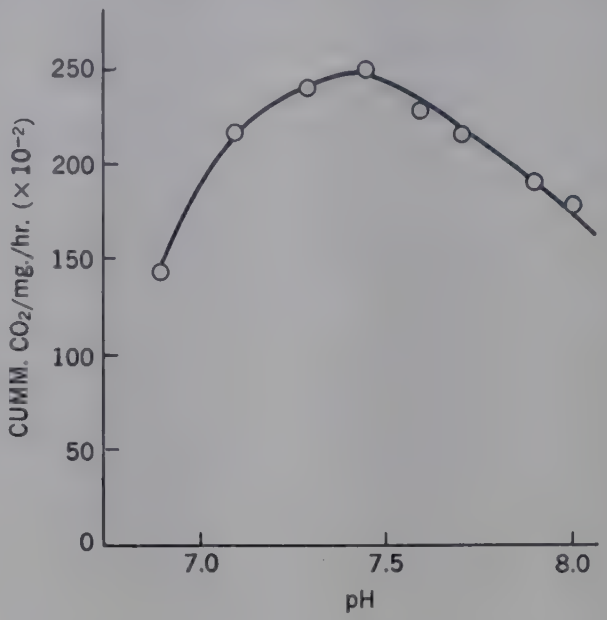

FIG. 6.

FIG. 5. Effect of variation of substrate level on ribonuclease activity. [From Bain and Rusch.[39]] Beginning with the highest, the curves represent 200, 150, 75, 50, and 25 mg. of substrate per flask, respectively.

FIG. 6. Effect of pH on the activity of ribonuclease I. [From Bain and Rusch.[39]] 3.0-ml. reaction mixture containing 200 mg. of sodium ribonucleate, and varying concentrations of sodium bicarbonate, together with 20 μg. crystalline ribonuclease.

The kinetic observations just discussed were made before the discovery of cyclic nucleotides as intermediaries of the hydrolysis of PNA by ribonuclease. It is obvious that the understanding of the kinetics of ribonuclease action requires supplementary data which will permit the interpretation of the earlier results in the light of the recent information on the reaction mechanism of the enzyme.

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[43] C. A. Zittle, *J. Biol. Chem.* **160,** 527 (1945).

Influence of pH. The pH optimum of crystallized pancreas ribonuclease was found by Kunitz[9] to be in the region of pH 7.7 (Fig. 6). Appreciable spontaneous degradation of ribonucleic acids occur already at this pH in experiments of longer duration. Many studies on ribonuclease action are therefore carried out at slightly acid pH values in the range between 5 and 6 in which the enzyme has still considerable activity.

Influence of Temperature. At pH 5, the optimal temperature of ribonuclease action is 65°.[9] In the range between pH 2 and pH 5 ribonuclease is rather heat-stable. Only about 20% of the activity is lost when ribonuclease is kept under these conditions at a temperature of 100° for thirty minutes.

Activators, Inhibitors. Ribonuclease I requires for its optimal activity an ionic strength of about 0.1. The activating effects of various univalent cations are quantitatively similar and larger than those observed with magnesium ions.[33,43a] Zittle observed that contamination of ribonucleates with copper ions impaired their susceptibility to ribonuclease action.[43, 44] Sodium fluoride even at 0.1 *M* concentration is without appreciable effect on ribonuclease. Zöllner and Fellig[45] showed good evidence for a specific competitive inhibition of ribonuclease activity by heparin. Some other acidic polysaccharides were without effect. Massart *et al.*[46] reported an inhibitory influence of penicillin on ribonuclease on the basis of a histochemical assay of enzyme activity.

Ledoux[47] studied the influence of the sulfhydryl reagent *p*-chloromercuribenzoate. He concluded that the complex influence of this substance resulted on the one hand from the inhibitory effect of its combination with the sulfur groups of the enzyme, on the other hand, from the activating influence of its combination with polar groups of the substrate. (See also [43a])

Ribonuclease as a Protein. The crystallization of ribonuclease was achieved by Kunitz[9] in 1940 by fractionation with ammonium sulfate. The enzyme prepared according to this procedure retains traces of contaminating proteolytic enzymes even after several recrystallizations.[48] Since crystalline ribonuclease is frequently applied as a histochemical reagent, an important advance was made when McDonald succeeded in removing these contaminations by introducing a heating step into the procedure of purification.[49] Labeled ribonuclease was prepared by Anfinsen[50] by incubating slices of beef pancreas in the presence of $C^{14}O_2$.

[43a] S. R. Dickman, R. B. Knopf, and J. B. Aboskar, *Abstr. 126th Meeting Am. Chem. Soc., New York* p. 71C (1954).

[44] The effects of copper ions can be abolished by versene.[43a]

[45] N. Zöllner and J. Fellig, *Am. J. Physiol.* **173,** 223 (1953).

[46] L. Massart, G. Peters, and A. Vanhauke, *Experientia* **3,** 494 (1947).

[47] L. Ledoux, *Biochim. et Biophys. Acta* **11,** 517 (1953).

[48] M. R. McDonald, *J. Gen. Physiol.* **32,** 33 (1948).

Despite the homogeneous behavior of ribonuclease during sedimentation and electrophoresis (Rothen[51]), recent studies of Martin and Porter[52] and of Hirs, Moore, and Stein[53] with ion-exchange chromatography demonstrated that crystalline ribonuclease, as well as fresh pancreas extracts, contains at least two ribonucleases (Ribonuclease IA and IB). Ledoux's[53a] suggestion that the chromatographic inhomogeneity of ribonuclease might be attributed to different stages of oxidoreduction of its sulfur groups is not in agreement with observations of other authors.[43a]

Chromatographically purified ribonuclease IA (the slower-moving band) was obtained in crystalline form, whereas the isolation of ribonuclease IB has so far not been achieved on the preparative scale. The assay methods used for the activity determinations on both enzymes were based on the formation of acid-soluble phosphorus compounds from yeast PNA. At present, no information regarding the finer enzymic specificities of the two pancreas ribonucleases is available.

All data regarding the physicochemical properties of ribonuclease are still based on measurements carried out in 1940 by Rothen[51] on ribonuclease samples prepared without chromatographic resolution into ribonucleases IA and IB. According to these measurements, the sedimentation rate of crystalline ribonuclease I in 0.5 M ammonium sulfate is $S^{25} = 1.85 \times 10^{-13}$. The diffusion coefficient in 0.5 M ammonium sulfate is $D = 1.36 \times 10^{-6}$. These data correspond to a molecular weight of 12,700, which is in good agreement with the mean value of 13,000 obtained from sedimentation equilibria by Rothen. Kunitz[9] arrived at the value of 15,000 (±1000) from osmotic pressure measurements. Most of the calculations in the current literature are based on the assumed value of 13,500. From the most recent amino acid analyses of Hirs, the value of 14,100 for the molecular weight of ribonuclease I is obtained.[53,53b,53c]

The isoelectric point of ribonuclease I was found at pH 7.8. The specific volume has the rather low value of 0.709. Ribonuclease I is a globular protein with a dissymmetry factor $\frac{f}{f_0} = 1.04$.

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[49] M. R. McDonald, *J. Gen. Physiol.* **32,** 39 (1948).

[50] C. B. Anfinsen, *J. Biol. Chem.* **186,** 827 (1950).

[51] A. Rothen, *J. Gen. Physiol.* **24,** 203 (1940).

[52] A. J. P. Martin and R. R. Porter, *Biochem. J.* **49,** 215 (1951).

[53] C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.* **200,** 493 (1953).

[53a] L. Ledoux, *Biochim. et Biophys. Acta* **14,** 267 (1954).

[53b] C. H. W. Hirs, *Federation Proc.* **13,** 230 (1954).

[53c] C. H. W. Hirs, W. H. Stein, and S. Moore, *Abstr. 126th Meeting Am. Chem. Soc., New York* p. 89C (1954).

Observations of Anfinsen *et al.*[54,55] suggest that ribonuclease I contains only one C terminal group (valine) and one N terminal group (lysine). The analysis of ribonuclease I by means of X-ray diffraction suggests the presence of five crystallographic chains per molecule.[56] Elliott[57] studied the infrared spectrum of single crystals of ribonuclease I and observed dichroism indicating the presence of folded polypeptide chains. According to Anfinsen, the available information suggests the working hypothesis that ribonuclease I consists of a single peptide chain which is folded to the five crystallographic chains postulated on the basis of the X-ray diffraction patterns of ribonuclease I crystals.[56] Possibly the four cystine groups[55,58-61] present in each molecule of ribonuclease are involved in maintaining the folded structure of the protein (Fig. 7). According to Anfinsen,[62] the enzymic activity of ribonuclease I is destroyed by digestion with crystallized pepsin. During the first phase of this digestion approximately 65% of the activity is lost without a measurable appearance of new end-groups and without a significant decrease of the sedimentation rate.[63] This slow phase

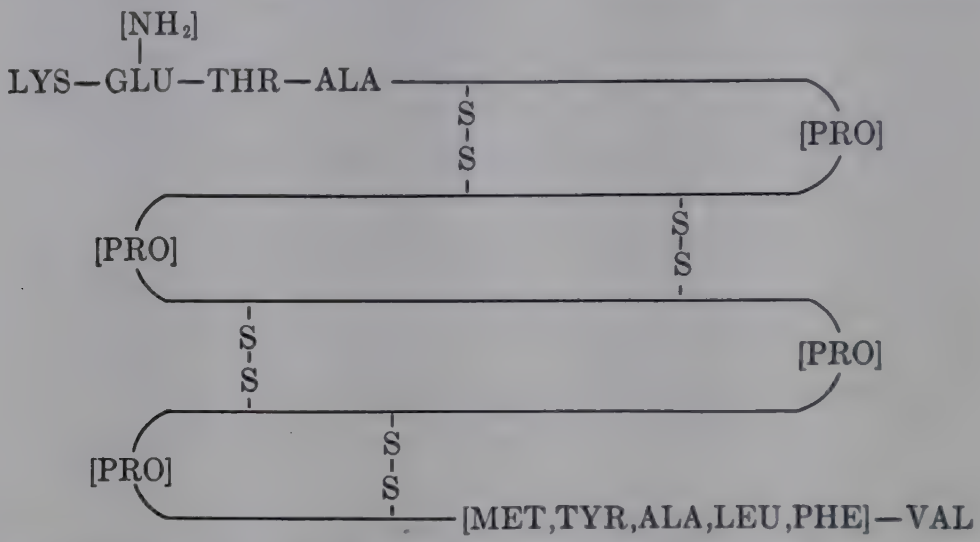

FIG. 7. Scheme of the shape of the molecule of ribonuclease I. [From Anfinsen *et al.*[55]] (The abbreviations are symbols for amino acids.)

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[54] C. B. Anfinsen, M. Flavin, and J. Farnsworth, *Biochim. et Biophys. Acta* **9,** 468 (1952).

[55] C. B. Anfinsen, R. R. Redfield, W. L. Choate, J. Page, and W. R. Carroll, *J. Biol. Chem.* **207,** 201 (1954).

[56] H. Carlisle and H. Scouloudi, *Proc. Roy. Soc. (London)* **A207,** 496 (1951).

[57] A. Elliott, *Proc. Roy. Soc. (London)* **A211,** 490 (1952).

[58] E. Brand, *Ann. N. Y. Acad. Sci.* **47,** 187 (1946).

[59] G. R. Tristram, *Advances in Protein Chem.* **5,** 145 (1949).

[60] J. H. Northrop, M. Kunitz, and R. M. Herriott, "Crystalline Enzymes," p. 26, New York, 1948.

[61] "Symposium on Mechanism of Enzyme Action" (W. D. McElroy and B. Glass, eds.). Johns Hopkins Press, Baltimore, 1953.

[62] C. B. Anfinsen, *J. Biol. Chem.* **196,** 201 (1952).

[63] D. Shugar, *Biochem. J.* **52,** 142 (1952).

is followed by a rapid deeper cleavage of the ribonuclease I accompanied by a practically total loss of activity. Hirs[53b] and Hirs, Stein, and Moore[53c] applied oxidation of ribonuclease with performic acid at −10° and subsequent digestion of the oxidized enzyme with trypsin to the investigation of the amino acid sequence in the ribonuclease molecule. The treatment of the protein with performic acid oxidized exclusively the sulfur groups of the cystine and methionine components. The number of polypeptides obtained by chromatography of the tryptic digest closely agreed with that predicted from the experimentally determined values of 10 lysine and 4 arginine rests for each molecule of ribonuclease.

b. *Other Ribonucleases*

The existence of ribonucleases other than the enzyme obtained in crystallized form from pancreas can now be considered as certain. They differ from pancreas ribonuclease in regard to their specificity toward the internucleotide bonds of the substrate, in regard to their heat lability and to their pH optima. Schmidt *et al.*[64] found in 1950 that incubation of PNA with crude pancreas extracts resulted in the cleavage of internucleotide bonds which were resistant to the action of crystallized pancreas ribonuclease I. These bonds were the ester linkages of 3′-purine nucleotide groups with the adjacent nucleotide groups. Maver and Greco[65] reported the presence in spleen extracts of a heat-labile ribonuclease which differed from ribonuclease I also by the different pH range (optimum in the region of pH 5.2, in the presence of Mg; at pH 6.6 without addition of Mg salts[66]) of its optimal activity.

Clear evidence for the existence of different ribonucleases and for the nature of their catalytic activity was obtained by Hilmoe and Heppel,[67] who fractionated the nucleolytic enzymes of spleen extracts. They studied the action of these enzymes not only on PNA, but on the limit polynucleotides obtained by exhaustive hydrolysis of PNA with ribonuclease I as well as on some simple synthetic nucleotide-P-esters of well-defined structure. Hilmoe and Heppel prepared from spleen a ribonuclease which was free from phosphomonoesterases and which hydrolyzed the limit polynucleotide fraction of PNA approximately four times faster than PNA itself. The pH optimum of this enzyme was at pH 6.6. The enzyme was rapidly inactivated at 60°.

Mechanism of Action. The spleen ribonuclease does not hydrolyze cyclic nucleotides. It is capable of catalyzing exchange reactions between nucleoside-containing diesters of phosphoric acid, such as:

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[64] G. Schmidt, R. Cubiles, and S. J. Thannhauser, *J. Cellular Comp. Physiol.* **38,** Suppl. 1, 61 (1950).

[65] M. E. Maver and A. E. Greco, *J. Biol. Chem.* **181,** 861 (1949).

[66] M. E. Maver and A. E. Greco, *Federation Proc.* **13,** 261 (1954).

[67] R. J. Hilmoe and L. A. Heppel, *Federation Proc.* **12,** 217 (1953).

2 cytidine-3′-benzylphosphate
= cytidylyl-3′,5′-cytidylyl-3′-benzylphosphate + benzyl alcohol

Ribopolynucleotidases of Plants. Bredereck *et al.*[68,69] found that aqueous extracts of sweet almonds, lucern seeds, or sprouted peas contain an enzyme system capable of converting ribonucleate practically completely to the nucleosides and phosphoric acid, at a pH range between 5 and 6. A fractionation of this system has so far not been attempted.

Bacterial Ribonucleases. Muggleton and Webb[70] found in culture filtrates of soil actinomyces (strain A) a very heat-labile ribonuclease, which was capable of hydrolyzing PNA as well as the polynucleotides resistant toward ribonuclease I. The presence of this ribonuclease is responsible for the power of the culture filtrates to render suspensions of heat-killed pneumococci gram-negative.

2. DEOXYRIBONUCLEASES

a. *Pancreas Deoxyribonuclease (Deoxyribonuclease I)*

History. Araki[70a] observed in 1903 that extracts of several tissues such as liver, spleen, and thymus had the power to liquefy gels of deoxyribonucleic acid. Abderhalden and Schittenhelm[71] (1906) as well as de la Blanchardière[71a] (1913) found that pancreatic juice of dogs effected this liquefaction without liberation of purine bases or inorganic phosphate. Feulgen[72,73] added the important observation that the degradation of deoxyribonucleic acid by pancreas preparations (pancreatin) did not yield mononucleotides, but stopped at the formation of oligonucleotides. Schmidt, Pickels, and Levene[74] found in 1938 that the degradation of DNA to oligonucleotides by the pancreas enzyme or by alkali was an essential intermediary step for the enzymic cleavage of DNA to mononucleotides (and subsequently to nucleosides) by the phosphatase of intestinal mucosa. Intestinal phosphatase does not act on highly polymerized DNA, but it hydrolyzes all interlinkages of the mononucleotide groups in the oligonucleotide mixture obtained by the action of deoxyribonuclease. Some-

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[68] H. Bredereck and G. Rothe, *Ber.* **71B,** 408 (1938).

[69] H. Bredereck, G. Caro, and F. Richter, *Ber.* **71B,** 2389 (1938).

[70] P. W. Muggleton and M. Webb, *Biochim. et Biophys. Acta* **9,** 343 (1952).

[70a] T. Araki, *Z. physiol. Chem.* **38,** 84 (1903).

[71] E. Abderhalden and A. Schittenhelm, *Z. physiol. Chem.* **47,** 452 (1906).

[71a] P. de la Blanchardière, *Z. physiol. Chem.* **87,** 291 (1913).

[72] R. Feulgen, "Chemie und Physiologie der Nucleinstoffe." Berlin, 1923.

[73] R. Feulgen, *Z. physiol. Chem.* **237,** 261 (1935).

[74] G. Schmidt, E. G. Pickels, and P. A. Levene, *J. Biol. Chem.* **127,** 251 (1939).

what earlier, Schmidt[75] had already found that fresh extracts of nucleohistone from thymus glands were not hydrolyzed by intestinal phosphatase, but that they were dephosphorylated by this enzyme after a preceding incubation with crude trypsin. This effect was undoubtedly due to the enzymic depolymerization of the nucleic acid component of the nucleohistone by the deoxyribonuclease present in the trypsin preparations, but not to proteolysis. Laskowski[76] (1946) and McCarty[77] achieved a considerable purification of the enzyme obtained from pancreas. The most essential contribution of this work was the separation of deoxyribonuclease from the powerful ribonuclease of pancreas and the evidence for the strict specificity of deoxyribonuclease for DNA. The crystallization of the enzyme which precipitates at much lower ammonium sulfate concentration (0.4 sat.) than does ribonuclease I (0.8 sat.) was achieved by Kunitz[3a] in 1950.

An interesting historical fact is the influence which the now abandoned working hypothesis of the tetranucleotide structure of nucleic acids had on the interpretation of the action of deoxyribonuclease. All investigators agreed that, after exhaustive incubation of DNA with deoxyribonuclease, about one out of four phosphoryl groups is present as a terminal secondary phosphoryl group. For a long time this observation was considered as evidence suggesting that the main products of the enzymic degradation were tetranucleotides and that, therefore, natural DNA should be considered as polymers of tetranucleotide units.[73] This view was predominant until, in 1951, chromatography was developed by Cohn and Volkin, and Smith and Markham, as a tool for the fractionation of oligonucleotide mixtures. We know now that there is no correlation between the relative amount of terminal phosphoryl groups and the chain length of the oligonucleotide units, and that deoxyribonuclease digests of DNA contain dinucleotides as well as hexanucleotides and oligonucleotides of other degrees of polymerization.

Specificity. The number of substrates tested for their behavior toward deoxyribonuclease is far less extensive than that studied with ribonuclease I. Nevertheless, the available observations justify the conclusion that deoxyribonuclease is a highly specific phosphodiesterase; in particular, it is without any effect on ribonucleic acids and ribopolynucleotides. In analogy to the action of ribonucleases, that of deoxyribonuclease I never results in the formation of inorganic phosphate. According to Tamm, Shapiro, and Chargaff,[78] the degree of polymerization of DNA is not of essential influence on the rate and on the extent of the action of deoxyribonuclease; partial or total removal of the purine groups, however, greatly diminishes the catalytic effects of the enzyme. It should be mentioned that Tamm *et al.* observed in the absence of deoxyribonuclease I degrading effects of the bivalent cations (Mg^{++}, Mn^{++}) required as activators of deoxyribonuclease I on the deoxypolynucleotides of lower molecular weights. These effects obviously render it difficult to evaluate accurately the action of deoxyribonuclease on DNA fragments of lower molecular weights. Little and Butler[79] isolated the nucleotide fraction formed by exhaustive enzymic degradation of highly polymerized (acid-insoluble) DNA to acid-soluble nucleotides. The titration of this fraction demonstrated the formation of secondary phosphoryl groups amounting to 25% of the total phosphoryl groups. This result is in agreement with earlier observations of Fischer, Böttger, and Lehmann-Echternacht.[80] Gordon and Reichard,[81] Sinsheimer and Koerner,[82,83,83a] as well as Smith and Markham[84] have succeeded recently in identifying by chromatography on paper and on ion-exchange columns some of the oligonucleotides as dinucleotides and trinucleotides. So far, deoxyadenyl-cytidylic, adenyl-thymidylic, guanyl-thymidylic, thymidylyl-thymidylic, cytidylyl-cytidylic, methylcytidylyl-cytidylic, methylcytidylyl-guanylic, adenyl-adenylic, adenyl-guanylic, guanyl-guanylic acids and a trinucleotide consisting of one thymidylic and two cytidylic acid groups were identified amongst the degradation products present in deoxyribonuclease digests of calf thymus DNA. Digests of wheat germ DNA contained methylcytidylyl-cytidylic and methylcytidylyl-adenylic acids in addition to the dinucleotides just mentioned. It is interesting that not all dinucleotides which have been analyzed up to now contain a pyrimidine component in contrast to the oligonucleotides formed from PNA by the action of ribonuclease. The amounts of mononucleotides formed by the action of deoxyribonuclease on DNA are much smaller than those formed by ribonuclease from PNA. The first evidence for the formation of mononucleotides by the hydrolysis of DNA with deoxyribonuclease I was obtained by Potter, Brown, and Laskowski,[85] who detected the appearance of appreciable quantities of deoxycytidylic acid in such digests. They represent only approximately 1% of the total phosphorus of the substrate.[83a]

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[75] G. Schmidt, *Enzymologia* **1**, 135 (1936).

[76] M. Laskowski, *Arch. Biochem.* **11**, 41 (1946).

[77] M. McCarty, *J. Gen. Physiol.* **32**, 39 (1948).

[78] C. Tamm, H. S. Shapiro, and E. Chargaff, *J. Biol. Chem.* **199**, 313 (1952).

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[79] J. A. Little and G. C. Butler, *J. Biol. Chem.* **188,** 695 (1951).

[80] F. G. Fischer, I. Böttger, and H. Lehmann-Echternacht, *Z. physiol. Chem.* **271,** 246 (1941).

[81] A. H. Gordon and P. Reichard, *Biochem. J.* **48,** 569 (1951).

[82] R. L. Sinsheimer and J. F. Koerner, *Science* **114,** 42 (1951).

[83] R. L. Sinsheimer and J. F. Koerner, *J. Am. Chem. Soc.* **74,** 283 (1952).

[83a] R. L. Sinsheimer, *J. Biol. Chem.* **208,** 445 (1954).

[84] J. D. Smith and R. Markham, *Biochim. et Biophys. Acta* **8,** 350 (1952).

[85] J. L. Potter, K. D. Brown, and M. Laskowski, *Biochim. et Biophys. Acta* **9,** 150 (1952).

In deoxyribonuclease digests of calf thymus DNA, thymidylic, cytidylic, adenylic, and guanylic acids were detected; in those of wheat germ DNA, methylcytidylic acid was found in addition to the other mononucleotides. The amounts of thymidylic acid accounted for 50 to 60% of the total mononucleotides formed.

Dinucleotides accounted for 15 to 18% of the substrate phosphorus. Although most of the dinucleotides contained a pyrimidine group, appreciable amounts of purine dinucleotides were found in deoxyribonuclease digests of calf thymus and wheat germ DNA.

From the fact that exclusively 5′-mononucleotides were obtained by the action of phosphodiesterase[84] on oligonucleotides obtained with deoxyribonuclease I, one can imply that the terminal secondary phosphoryl groups of these oligonucleotides are attached to the 5′-positions of the corresponding deoxyribose moieties.

An appreciable portion of the higher oligonucleotides of deoxyribonuclease I digests is not dialyzable against water. This undialyzable fraction was designated by Zamenhof and Chargaff[86] as "cores." These cores have much higher adenine:guanine, thymine:cytosine, and purine:pyrimidine ratios than are found in the original substrate. The possibility of a correlation between the sequence of the bases in DNA and the points of attack of deoxyribonuclease is not yet clear, but it appears that some seeming resemblance of deoxyribonuclease and ribonuclease action in this respect does not justify the assumption of similar underlying specificities.

The effect of deoxyribonuclease on the viscosity of DNA has recently been used for some therapeutic purposes.[86a]

Assay Methods. It is obvious that some of the tests for deoxyribonuclease activity resemble in principle those for ribonuclease activity and need not be discussed in detail. *Formation of acidic groups*: The transformation of primary into secondary phosphoryl groups can be measured by titration or manometric methods. The conditions for such measurements with DNA as substrate are more favorable than similar determinations of ribonuclease activity because of the negligible amounts of preformed secondary phosphoryl groups in highly polymerized DNA. *Formation of acid-soluble phosphorus or deoxyribose compounds*: The merits of these assay methods have been discussed by Laskowski,[76] Kurnick,[87] and Allfrey and Mirsky.[87a] The determination of acid-soluble P compounds in deoxyribonuclease digests is likewise more accurate than that in ribonuclease digests owing to the practically complete insolubility of DNA even in very dilute solutions. The colorimetric determination of acid-soluble deoxyribose compounds by means of Dische's reagent is particularly convenient according to Allfrey and Mirsky since the time-consuming ashing procedure required for determination of the acid-soluble phosphorus is eliminated. *Optical changes*: Kunitz[3a] found that the action of deoxyribonuclease on DNA is accompanied by an increase of absorption at 260 mμ amounting maximally to 30%. Under standard conditions, the initial increase per minute of the optical density is proportional to the amounts of deoxyribonuclease, and its determination is a useful assay method particularly for purified preparations of deoxyribonuclease. Similar optical changes were observed by Kurnick[88] during the nonenzymic depolymerization of DNA by heat. *Viscosity changes*: The hydrolysis of DNA is accompanied by a rapid decrease of the viscosity of the enzyme–substrate mixtures. This phenomenon is a useful basis for enzyme determinations since the initial rates of decrease are proportional to the amounts of deoxyribonuclease.[75,89-91] Since the viscosity of DNA solutions is influenced by various other factors, such assays must be carried out under strictly comparable conditions, particularly in regard to electrolyte concentrations. *Binding of methyl green* (*Kurnick's method*):[87,92] Kurnick found that methyl green (ethylated hexamethylpararosaniline) combines only with highly polymerized DNA, and that the color of this compound is stable at pH 7.5 in contrast to free methyl green which fades at this pH. When DNA containing methyl green is used as substrate solution, the depolymerization of DNA by deoxyribonuclease (or by heat) is accompanied by the liberation of a corresponding amount of methyl green. The rate of decrease of absorption at 640 mμ at pH 7.5 can be used as a measure of deoxyribonuclease activity. Since the fading of the color of the "liberated" methyl green is not instantaneous, the enzyme action is stopped by the addition of citrate, and the colorimetric readings are carried out after four hours' standing.

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[86] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **187,** 1 (1950).

[86a] J. N. Davidson, *Brit. Med. Bull.* **9,** 154 (1953).

[87] N. B. Kurnick, *Arch. Biochem.* **29,** 41 (1950).

[87a] V. G. Allfrey and A. E. Mirsky, *J. Gen. Physiol.* **36,** 227 (1952).

[87b] S. G. Laland, W. A. Lee, W. G. Overend, and A. R. Peacocke, *Biochim. et Biophys. Acta* **14,** 356 (1954).

The behavior of another basic dye, methylene blue, during the action of deoxyribonuclease on DNA was studied by Vercauteren.[93-95] The binding of this dye, unlike that of methyl green, does not require a very high degree of polymerization of DNA; its liberation (as measured by the appearance of methylene blue in the supernatant solution after the precipitation of the dye–polynucleotide compounds with acid) begins only in the later phases of deoxyribonuclease action. The characteristic initial drop of viscosity is therefore not accompanied by the liberation of methylene blue. This interpretation is in analogy with the observations of Jungner *et al.*,[96,97] who found that the dielectric constants of deoxyribonuclease digests remained unchanged during phases of rapid decreases of viscosity.

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[88] N. B. Kurnick, *J. Gen. Physiol*, **33**, 243 (1950).

[89] J. P. Greenstein and W. V. Jenrette, *Cold Spring Harbor Symposia Quant. Biol*. **9**, 236 (1941).

[90] J. P. Greenstein, *J. Natl. Cancer Inst.* **2**, 357 (1942).

[91] M. McCarty, *J. Gen. Physiol.* **29**, 123 (1946).

[92] N. B. Kurnick, *Arch. Biochem. and Biophys.* **43**, 97 (1953).

[93] R. Vercauteren, *Nature* **165**, 603 (1950).

[94] R. Vercauteren, *Enzymologia* **14**, 134 (1950).

[95] R. Vercauteren, *Arch. intern. physiol.* **57**, 214 (1949).

Most methods of assay yield satisfactory results with purified deoxyribonuclease preparations but are not easily adaptable to enzyme assays in crude tissue extracts or serum. Kurnick's procedure, however, is applicable to serum. It should be mentioned that Alfert reported evidence suggesting that changes of methyl green binding by DNA can be caused by factors other than depolymerization.[98]

Kinetics. The kinetics of the degradation of DNA by deoxyribonuclease I is obviously an overall picture of many different individual enzyme processes. No simple substrates for deoxyribonuclease are available as yet.

At low concentrations of DNA (160 μg. per ml.) the hydrolysis curve follows the course of a pseudounimolecular reaction, and the velocity constants are proportional to the concentrations of deoxyribonuclease in the digestion mixture.[99] At high concentrations of the substrate, the hydrolysis curve is an asymmetric S-shaped curve owing to a short initial lag period.[3a]

Activators. Unlike ribonuclease I, deoxyribonuclease requires for its action the presence of bivalent cations.[3a,76,88,91,100-105] Magnesium, manganese, ferrous, and cobaltous ions are the most effective activators and resemble each other in regard to the quantitative intensity of activation. The observations regarding the effects of some other cations, such as zinc and calcium, are contradictory.[106] The concentration required for optimal effects of the activating cations is practically independent of the enzyme concentration but increases with increasing substrate concentrations.

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[96] G. Jungner, I. Jungner, and L. G. Allgén, *Nature* **163,** 849 (1949).

[97] G. Jungner, *Acta Physiol. Scand.* **10,** Suppl. 32, 1 (1945).

[98] M. Alfert, *Biol. Bull.* **103,** 145 (1952).

[99] J. Grégoire, *Compt. rend.* **231,** 384 (1950).

[100] F. G. Fischer, H. Lehmann-Echternacht, and I. Böttger, *J. prakt. Chem.* **159,** 59 (1941).

[101] M. Laskowski and M. K. Seidel, *Arch. Biochem.* **7,** 465 (1945).

[102] C. E. Carter and J. P. Greenstein, *J. Natl. Cancer Inst.* **7,** 29 (1946).

[103] J. P. Greenstein, C. E. Carter and H. W. Chalkley, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 64 (1947).

[104] W. G. Overend and M. Webb, *Research* **2,** 99 (1949).

[105] T. Miyaji and J. P. Greenstein, *Arch. Biochem. and Biophys.* **32,** 414 (1951).

[106] L. M. Gilbert, W. G. Overend, and M. Webb, *Exptl. Cell Research* **2,** 349 (1951).

Optimal activation by magnesium ions was observed at a molar proportion of 3 between the magnesium ions and the total phosphorus of the substrate. Increases of the total salt concentration of the incubation mixture beyond 0.02 *M* are progressively inhibitory.

The observations just reported suggest that the salt activation of deoxyribonuclease is produced by the combination of the ions with the substrate and not by the formation of an enzyme-activator compound.[107,108]

General Stability and Influence of Heat. Solutions of deoxyribonuclease of relatively high concentration (0.1 mg. of enzyme protein per ml. of buffer solutions) retain their activities for several days at 5°. Highly dilute solutions, however, require the presence of stabilizing colloids (gelatin, peptones) for preservation.

When solutions of deoxyribonuclease I are kept at 90° for 5 minutes at pH 2.8, the activity disappears completely, but most of the original activity is restored by standing at 20° for 30 hours.

Inhibitors. (1) *Substances removing activating bivalent cations.* The requirement of certain bivalent cations for the activity of deoxyribonuclease I explains the inhibitory effect of certain anions such as citrate and fluoride on this enzyme.

(2) *Other inhibitors.* Copper, selenite, and arsenate ions are strongly inhibitory at 0.001 *M*, borate at 0.02 *M*. Hydroxylamine, cysteine, and iodoacetic acid have no appreciable effects. Mitotic poisons, such as colchicine and aminopterine, and SH-reactants, such as iodoacetamide and chloroacetophenone, caused no inhibition of the action of deoxyribonuclease I on deoxyribonucleohistone. Incubation of nucleohistone with nitrogen mustards resulted in a diminished susceptibility of the nucleoprotein toward deoxyribonuclease.

(3) *Specific inhibitors of deoxyribonuclease in tissues.* Tissue inhibitors of deoxyribonucleases are of great biological interest because of their possible regulatory function in the DNA metabolism of growing cells.[109] Recently, protein inhibitors of deoxyribonuclease I were found in several tissues. Dabrowska, Cooper, and Laskowski purified such a protein from growing pigeon crop glands.[110] On the basis of kinetic studies, Laskowski and his associates concluded that this effect was caused by a reversible association of a specific protein with deoxyribonuclease I. The inhibitor protein is easily extractable by water from the tissue, and is precipitated by ammonium sulfate between 0.3 and 0.6 saturation. It is destroyed by heat and by the action of trypsin. Protein fractions with similar inhibitory effects on

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[107] N. Weissman and J. Fisher, *J. Biol. Chem.* **178,** 1007 (1949).

[108] V. S. Shapot, *Biokhimiya* **17,** 290 (1952).

[109] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **180,** 727 (1949).

[110] W. Dabrowska, E. J. Cooper, and M. Laskowski, *J. Biol. Chem.* **177,** 991 (1949).

deoxyribonuclease I were also found in other animal tissues and in certain malignant tumors. In the pigeon crop glands, the concentration of the inhibitor protein is strongly increased during the hypertrophy of the epithelium occurring in glands of brooding birds; in other animal tissues, however, no definite correlation between inhibitor concentrations and cell growth could be established so far. Cooper, Trautman, and Laskowski demonstrated the presence of inhibition of deoxyribonuclease I in various mammalian tissues.[111] Henstell and Freedman[112] reported considerable inhibitory influence of extracts from mature white blood cells on pancreas deoxyribonuclease I. The concentration of this inhibiting activity was greatly diminished in white blood cells of leukemia patients[113] provided that immature cells were predominant in the blood. Kurnick *et al.*[114] correlated changes of the inhibiting activity of the white blood cells with the characteristic appearance of the L. E. cells in the blood and bone marrow of patients with lupus erythematosus.[115] McCarty demonstrated specific antigenic effects of purified preparations of pancreas deoxyribonuclease.[116] Bernheimer and Ruffier[117] found that extracts of streptococci contained an inhibitor of pancreas deoxyribonuclease I, the activity of which was destroyed by the action of ribonuclease.[117]

pH Optimum. Deoxyribonuclease I has maximal activity between pH 6 and pH 7. The precise values of the pH optimum differ somewhat for different kinds of activating bivalent cations.[105]

Deoxyribonuclease I as a Protein. Owing to the small yields (Kunitz obtained from 10 kg. of pancreas approximately 20 mg. of the enzyme), information regarding the chemical properties of deoxyribonuclease I is much less extensive than the corresponding data available for crystallized ribonuclease.

The molecular weight of deoxyribonuclease I (based on its diffusion in the Northrop-Anson cell) was found by Kunitz[3a] to be approximately 60,000. It contains 8% of tyrosine and 2% of tryptophan. The ultraviolet absorption of deoxyribonuclease I is a consequence of the presence of these two amino acids in the molecule.

The pH of minimal electrophoretic mobility is approximately 5.

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[111] E. J. Cooper, M. L. Trautmann, and M. Laskowski, *Proc. Soc. Exptl. Biol. Med.* **73,** 219 (1950).

[112] H. H. Henstell and R. L. Freedman, *Cancer Research* **12,** 341 (1952).

[113] H. H. Henstell, R. L. Freedman, and B. Ginsburg, *Cancer Research* **12,** 346 (1952).

[114] N. B. Kurnick, L. I. Schwartz, S. Pariser, and S. L. Lee, *J. Clin. Invest.* **52,** 193 (1953).

[115] S. L. Lee, S. R. Michael, and I. L. Vural, *Am. J. Med.* **10,** 446 (1951).

[116] M. McCarty, *J. Gen. Physiol.* **29,** 123 (1946).

[117] A. W. Bernheimer and N. K. Ruffier, *J. Exptl. Med.* **93,** 399 (1951).

b. Other Deoxyribonucleases

Recent observations from several laboratories demonstrated the existence of several different enzymes catalyzing the cleavage of the internucleotide bonds of DNA.[118]

Maver and Greco[65,66] and Brown, Jacobs, and Laskowski[119] found that the considerable deoxyribonuclease activity of thymus glands is largely due to the presence of an enzyme which can be differentiated from deoxyribonuclease I. Laskowski and his collaborators[119-121] as well as Webb[122,123] succeeded in purifying the extractable fraction of thymus deoxyribonuclease several hundred fold. The enzyme was inhibited by 0.025 *M* magnesium sulfate, but it was not affected by 0.005 *M* magnesium sulfate. Its activity was not influenced by the specific protein inhibitor of deoxyribonuclease I. Its pH optimum was at pH 5.

Thymus deoxyribonuclease II hydrolyzes DNA without the formation of undialyzable cores.

The association of the tissue deoxyribonucleases with particulate fractions[87a,123] is discussed in Section X of this Chapter.

Deoxyribonucleases of Plants. The occurrence of deoxyribonucleases in plants has been reported,[124,125] but very little detailed information concerning the properties of these enzymes is as yet available. The high concentrations of deoxyribonuclease activity in various germinating seeds[109,124] are of particular interest.

Deoxyribonucleases of Microorganisms. Various microorganisms[109,126-130] contain highly active deoxyribonucleases. Only a few of these enzymes have so far been studied in some detail. Zamenhof and Chargaff[109] obtained from extracts of bakers' yeast a complex of a deoxyribonuclease with a specific inhibitor protein by precipitation in 0.6 saturated ammonium sulfate solution. On prolonged autolysis at low temperature, the inhibitor protein is destroyed by proteolytic enzymes, and the deoxyribonuclease activity increases correspondingly, e.g., during 90 days' autolysis, the deoxyribonuclease activity of a yeast extract increased fifty fold according to the results of a viscosimetric assay procedure. The deoxyribonuclease differs from deoxyribonuclease I of pancreas by its pH optimum which is in the region of pH 6. It is insoluble in water but soluble in dilute salt solution. It requires magnesium ions for its activity. The protein inhibitor which is destroyed by various crystallized proteolytic enzymes is specific for the yeast enzyme and has no activity toward deoxyribonuclease I, and toward deoxyribonucleases of thymus and of neurospora.

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[118] G. Herbert, K. Lang, and A. Corbet, *Biochem. Z.* **320,** 418 (1950).

[119] K. D. Brown, G. Jacobs, and M. Laskowski, *J. Biol. Chem.* **194,** 445 (1952).

[120] M. Laskowski, E. A. Steberl, R. Akka, and P. Watson, *Biochim. et Biophys. Acta* (1954), in press.

[121] M. Privat de Garilhe and M. Laskowski, *Biochim. et Biophys Acta* (1954), in press.

[122] M. Webb, *Exptl. Cell Research* **5,** 27 (1953).

[123] M. Webb, *Exptl. Cell Research* **5,** 16 (1953).

[124] J. P. Greenstein, *Federation Proc.* **1,** 113 (1942).

[125] G. Brawermann and E. Chargaff, *J. Biol. Chem.* **210,** 445 (1954).

[126] H. Plenge, *Z. physiol. Chem.* **39,** 190 (1903).

[127] M. McCarty and O. T. Avery, *J. Exptl. Med.* **83,** 97 (1946).

[128] S. S. Cohen, *J. Biol. Chem.* **168,** 511 (1947).

[129] W. S. T. Tillett, S. Sherry, and L. R. Christensen, *Proc. Soc. Exptl. Biol. Med.* **68,** 184 (1948).

[130] W. C. Schneider and C. H. Hogeboom, *J. Biol. Chem.* **198,** 155 (1952).

Muggleton and Webb[70] found in culture filtrates of a soil actinomyces (strain A) a very heat-labile deoxyribonuclease which hydrolyzed DNA and the cores obtained from digests of DNA with deoxyribonuclease I.

3. "Unspecific" Phosphodiesterases

a. Snake Venom Phosphodiesterases

The internucleotide bonds of PNA and of all deoxyribo- and ribonucleotides which do not contain terminal secondary phosphoryl groups in the 3′-position are hydrolyzed by purified extracts of the venoms of a variety of poisonous snakes, e.g., those of Crotalus, of Bothrops, of the Japanese snake "Habu." Since none of these enzymes has been obtained in pure, crystalline form, it is not possible at present to decide as to whether the phosphodiesterases of various snake venoms are identical or not. Historically, the snake venom phosphatases, whose action on nucleic acids was discovered in 1919 by Délézenne and Morel,[131] are of interest because Akamatsu's concept[132] (1931) of the existence of specific enzymes for the cleavage of phosphomonoesters and of phosphodiesters originated from observations with this group of enzymes. Crude snake venoms with few exceptions contain mixtures of phosphodiesterases and phosphomonoesterases. Hurst and Butler[133] succeeded, however, in separating both types of phosphatases chromatographically; Sinsheimer and Koerner[134] described a procedure involving alcohol fractionation for the preparation of phosphodiesterase containing only negligible quantities of phosphomonoesterases.

Specificity. Snake venom phosphodiesterase hydrolyzes not only oligonucleotides of the structures defined, but also diphenyl phosphate and *bis-p*-nitrophenyl phosphate to the corresponding monoesters and phenols without the formation of inorganic phosphate. It can be assayed by phenol determination or, in the case of the phosphodiesters of nitrophenols which are practically colorless, by direct photometric determination of the yellow color of the liberated nitrophenol.

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[131] C. Délézenne and H. Morel, *Compt. rend.* **168,** 241 (1919).

[132] T. Uzawa, *J. Biochem.* (*Japan*) **15,** 19 (1932).

[133] R. O. Hurst and G. C. Butler, *J. Biol. Chem.* **193,** 91 (1951).

[134] R. L. Sinsheimer and T. J. Koerner, *J. Biol. Chem.* **198,** 293 (1952).

Monophenyl phosphate, *p*-nitrophenyl phosphate, the glycerophosphates and all mononucleotides are resistant to the enzyme.

Nature of Degradation Products. Incubation of PNA as well as of deoxyribonuclease I digests of DNA results in the cleavage of practically all internucleotide bonds. (The appearance of small amounts of inorganic phosphate in many digests originates most likely from small contaminations of the phosphodiesterase preparations with phosphomonoesterases.) The action of snake venom phosphodiesterase on PNA was first studied by Gulland and Jackson,[135] who found that such digests contained phosphoryl groups which were hydrolyzed by a specific 5′-nucleotidase (see below) and concluded that a considerable amount of 5′-phosphoryl groups must be present in PNA itself. The action of this enzyme on PNA was convincingly elucidated in the investigations of Cohn and Volkin.[136] In the case of deoxyribooligonucleotides, the final hydrolysis products are exclusively the four 5′-mononucleotides. The exhaustive digestion of PNA with snake venom phosphodiesterase results in a mixture of the four 5′-nucleotides, of pyrimidine-2′,5′- and -3′,5′-diphosphomononucleotides, and of an amount of purine nucleosides approximately equivalent to that of the diphosphonucleotides.

Thus, in contrast to the action of ribonuclease, that of snake venom diesterase causes specifically the cleavage of the 3′-ester linkages between the phosphoryl groups and the corresponding nucleoside residues. The structural implications of these observations have been discussed in Chapter 12.

The practically complete cleavage of PNA and of all deoxyribooligonucleotides to mononucleotides or—in the case of PNA—to mixtures of mononucleotides and mononucleotide phosphate esters leads to the conclusion that all internucleotide bonds in these polynucleotides regardless of their structural differences are cleaved by snake venom phosphodiesterase. However, this general statement requires one qualification: Only those compounds are hydrolyzed by the enzyme which do not have a secondary phosphoryl group in the 3′-position. Thus, dinucleotides of the type shown in the lower part of Figure 8 are resistant to the enzyme, but nucleotide-nucleoside esters originating from dinucleotides by the removal of the terminal phosphoryl group by a phosphomonoesterase are hydrolyzed by snake venom diesterase.

Consequently, all oligonucleotides of deoxyribonuclease I-digests are

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[135] J. M. Gulland and E. M. Jackson, *Biochem. J.* **32,** 597 (1938).

[136] W. E. Cohn and E. Volkin, *J. Biol. Chem.* **203,** 319 (1953).

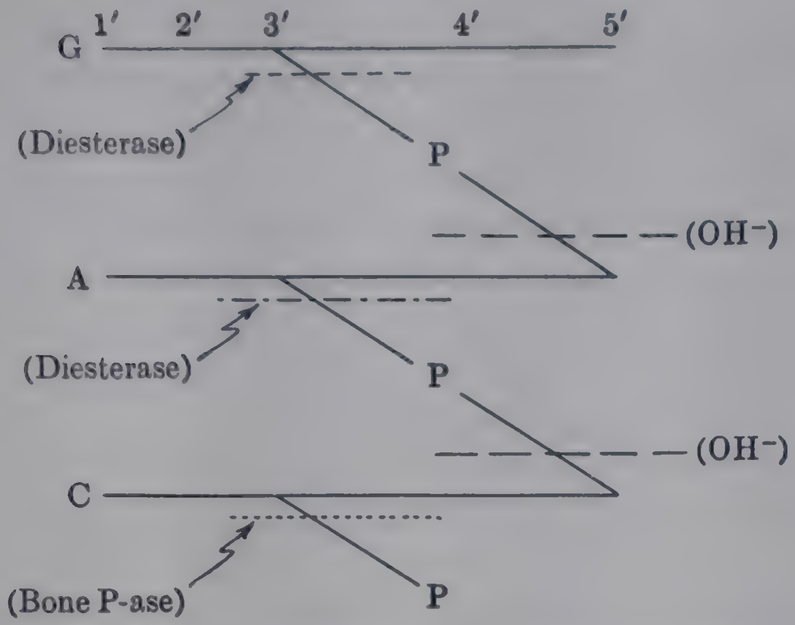

FIG. 8. Action of the phosphodiesterases of intestinal mucosa and of snake venom, and of bone phosphomonoesterase on PNA. [From Volkin and Cohn.[17]]

hydrolyzed by snake venom phosphodiesterase because their terminal phosphomonoester groups are in the 5′-position; on the other hand, the oligonucleotides of digests of PNA with ribonuclease I which contain the terminal secondary phosphoryl groups in the 3′-positions are hydrolyzed by snake venom phosphodiesterase only after the removal of these groups by a preliminary digestion with a phosphomonoesterase (bone phosphomonoesterase or prostatic phosphatase).

The question remains why undegraded PNA (prepared by mild procedures in the laboratory) is hydrolyzed by the enzyme without a preceding monoesterase treatment[136] despite its content of terminal 3′-phosphoryls which amounts to about 6 to 10% of its total phosphorus. Possibly the inhibiting influence of these terminal groups decreases with increasing degrees of polymerization of the substrates.

The resistance of highly polymerized deoxyribonucleic acids toward snake venom diesterase could be attributed either to their molecular size or to structural reasons. No decision between these alternatives is possible at present.

It may well be that the action of the snake venom enzyme is a simple hydrolysis; at least it is obvious that its action on DNA cannot involve an intermediary formation of 2,3-cyclic nucleotide groups.

Properties of Snake Venom Phosphodiesterases

Correlation with toxicity. According to Taborda *et al.*,[137] the average phosphodiesterase concentration in venoms of various species shows a significant parallelism with their toxicity. The activity of these enzymes is strongly inhibited by some factors known to depress the toxicity, such as antivenom serum, cysteine, and formaldehyde. The activity determinations of Taborda's group were carried out on crude extracts; since a manometric method was used, the values obtained represent figures of the phosphodiesterase activities.

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[137] A. R. Taborda, L. C. Taborda, J. N. Williams, Jr., and C. A. Elvehjem, *J. Biol. Chem.* **195,** 207 (1952).

pH Optimum. The optimum for the hydrolysis of PNA and of deoxyoligonucleotides is at pH 9.2.[134,136] Gulland and Jackson reported optimum hydrolysis of diphenyl phosphate at pH 8.6.[135]

Activators and inhibitors. Snake venom phosphodiesterase is considerably activated by 0.003 *M* magnesium ions, and to a lesser extent by manganese ions. Its activity seems to be inhibited by the characteristic inhibitors of alkaline phosphomonoesterases such as cyanide and cysteine, but the evaluation of the available data is difficult because of the high concentrations at which the inhibitors were studied. Adrenocorticotropic hormone and cortisone, both of which are known to alleviate the clinical toxicity of the venoms, has no effect on their phosphodiesterase activity.

b. "Phosphodiesterase" of Intestinal Mucosa

The studies of the enzymic cleavage of nucleic acids into lower polynucleotides and mononucleotides by duodenal juice or by extracts of intestinal mucosa were of great historical importance in the development of the chemistry of the nucleic acids. The use of this enzyme for the partial hydrolysis of nucleic acids in the laboratory of P. A. Levene[138] and in that of S. J. Thannhauser[139] was essential for the successful isolation of the nucleosides and nucleotides of deoxyribonucleic acids and for the isolation of D-deoxyribose. After the advent of ion-exchange chromatography, Cohn and Carter[140] succeeded for the first time in isolating the four 5′-ribonucleotides as products of the enzymic hydrolysis of PNA by intestinal phosphodiesterase.

Specificity. Unlike snake venom phosphodiesterase, intestinal phosphodiesterase has not been obtained free from phosphomonoesterase activity by fractionation methods.[67, 141] The phosphodiesterase activity of highly purified preparations of intestinal phosphatase is specific toward the bonds between the nucleotide groups of PNA and those of DNA polynucleotides of relatively low molecular weights. Cyclic 2′,3′-mononucleotides are not hydrolyzed by intestinal phosphodiesterase.[11] Diphenyl phosphate, glycerylphosphorylcholine, and glycerylphosphorylethanolamine are hydrolyzed by homogenates and crude autolysates of intestinal mucosa of calves, but not by highly purified intestinal phosphatase.[16]

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[138] P. A. Levene and L. W. Bass, "Nucleic Acids." Chemical Catalog Co., New York, 1931.

[139] W. Klein and S. J. Thannhauser, *Z. physiol. Chem.* **231**, 96 (1935).

[140] W. E. Cohn and C. E. Carter, *Nature* **167**, 483 (1951).

[141] G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.* **149**, 369 (1943).

Highly polymerized DNA is likewise resistant toward purified intestinal phosphatase,[74, 75] but digests of DNA obtained by incubation with deoxyribonuclease I are rapidly and completely hydrolyzed to nucleosides and inorganic phosphate.

Yeast and pancreas PNA are completely cleaved to nucleosides and orthophosphate by highly purified intestinal phosphodiesterase provided the incubation is carried out with relatively large amounts of the enzyme. The rates of hydrolysis of DNA polynucleotides by intestinal phosphatase are considerably higher than those of PNA.[142]

The hydrolysis of phosphomonoesters by intestinal phosphatase proceeds much more rapidly than that of polynucleotides; since the latter is thus the limiting factor, mononucleotides are usually not detectable in the digests. Klein and Thannhauser[143] found, however, that the phosphomonoesterase activity of intestinal mucosa is inhibited by arsenate to a higher degree than is its phosphodiesterase action. In the presence of arsenate, appreciable amounts of mononucleotides accumulate in the digests. The amounts of deoxymononucleotides in arsenate-containing digests of depolymerized DNA are sufficient to permit their isolation by fractionation procedures. The presence of mononucleotides in PNA hydrolysates obtained with intestinal phosphatase in the presence of arsenate was demonstrated by Cohn and Volkin by means of ion-exchange chromatography.[140,144] In contrast to pancreas ribonuclease I, which forms only 3′-pyrimidine nucleotides, the nucleotides originating from the action of intestinal phosphatase are 5′-purine and pyrimidine nucleotides.

The strong nucleophosphodiesterase activity of intestinal phosphatase raises the question as to whether alkaline phosphatases of other tissues are likewise capable of hydrolyzing certain phosphodiester linkages. So far, the alkaline phosphatases in various tissues have been considered as identical although Gulland[145] considered alkaline bone phosphatase as a strict phosphomonoesterase. On the strength of Gulland's interpretation, this enzyme has recently been used as a reagent for terminal secondary phosphoryl groups of nucleic acids.[136] The author of this review, however, is not convinced of the evidence in favor of the exclusive monophosphoesterase nature of bone phosphatase. Gulland's view was based on experiments with relatively weakly active phosphatase preparations, whereas the action of intestinal phosphatase was studied on highly active phosphatase samples. Owing to the relatively slow rate of the enzymic depolymerization of PNA and of deoxypolynucleotides, the diesterase action of weakly active phosphatase preparation might have been overlooked. It seems that the problem of nucleophosphodiesterase action of alkaline phosphatases requires clarification by further investigations.

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[142] W. Klein, *Z. physiol. Chem.* **207,** 164 (1933).

[143] W. Klein and S. J. Thannhauser, *Z. physiol. Chem.* **218,** 164 (1933).

[144] W. E. Cohn, D. G. Doherty, and E. Volkin *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 339. Johns Hopkins Press, Baltimore, 1952.

[145] J. M. Gulland and E. M. Jackson, *J. Chem. Soc.* **1938,** 1492.

pH Optimum. The pH optimum of intestinal nucleophosphodiesterase has been reported to be in the alkaline range; since all available observations are based on the liberation of inorganic phosphate from the substrate, it is possible that these data describe the behavior of the phosphomono- rather than that of the phosphodiesterase activity.

Assay. Approximate figures for the activities of intestinal phosphodiesterases are usually obtained by the determination of inorganic phosphate or of phenols which originate from the intermediary phosphomonoesters by the action of the phosphomonoesterase which is always present. In the case of other nucleophosphodiesterases, it might be preferable to determine the organic acid-soluble phosphorus compounds formed, particularly for studies of the effects of enzyme inhibitors.

III. Enzymes Catalyzing the Hydrolytic Cleavage of the Phosphoryl Groups of Mononucleotides

1. Phosphatases of Low Degrees of Specificity

Mononucleotides are rapidly dephosphorylated by many phosphatases such as intestinal phosphatase, bone phosphomonoesterase, acid prostatic phosphatase, almond phosphatase. Since these phosphatases are active toward many other phosphoric acid monoesters, their detailed description is beyond the scope of this book. It should be mentioned, however, that in some cases the hydrolysis of nucleotides shows kinetic differences in comparison with that of other substrates. The rates of hydrolysis of 3′-nucleotides by prostate phosphatase follow the course of a unimolecular reaction, at least up to degrees of 70% hydrolysis, whereas those of the hydrolysis of many other substrates (in particular, of the glycerophosphates) fall much more rapidly even in the initial stages.[37]

The phosphatases just mentioned hydrolyze all mononucleotides regardless of the position of the phosphoryl groups. In some cases, however, the rates of hydrolysis catalyzed by one enzyme are very markedly influenced by the position of the phosphoryl groups in the nucleoside moiety. For example, the hydrolysis of 5′-adenylic acid by prostatic phosphatase is approximately one-third of that of 2′- or of 3′-adenylic acid.[37]

2. Specific Nucleotide Phosphatases (Nucleotidases)

a. 5′-Nucleotidases

The existence of specific phosphatases for the hydrolysis of 5′-nucleotides was implicitly suggested by the old observation that mammalian skeletal muscle and heart tissue which do not hydrolyze glycerophosphate, 2′- and 3′-nucleotides, and many other monoesters of phosphoric acid, are capable of hydrolyzing 5′-adenylic and 5′-inosinic acids. Reis[146-148] established in systematic studies the widespread occurrence in mammalian tissues of a specific 5′-nucleotidase which is particularly abundant in muscle and nerve tissue and in bull testicle.[149] In these tissues, the largest part of the enzyme is closely associated with the insoluble particles.

In the cytoplasm and the nuclei of the cells of smooth muscle (such as uterus and aorta) and in fibroblasts, the presence of a specific 5′-nucleotidase was demonstrated histochemically by Wachstein and Meisel.[150] Particularly high concentrations of 5′-nucleotidase were found histochemically in the posterior lobe of the human pituitary gland.[151]

Soluble 5′-nucleotidases of strong activity were found in snake venoms. In the plant kingdom, potatoes are a convenient source for the preparation of purified extracts with highly specific 5′-nucleotidase activity.[152] The question as to whether the 5′-nucleotidases obtained from the various sources are closely related in regard to their chemical properties or not, cannot be decided at present because the purification procedures of these enzymes were designed for the purpose of obtaining preparations with high enzymic specificity rather than homogeneous enzyme proteins.

Properties of 5′-Nucleotidase of Bull Semen. Some of the properties of 5′-nucleotidase from bull semen, which was studied by Heppel and Hilmoe,[153] are as follows.

pH Optimum. Seminal 5′-nucleotidase has its optimal activity at pH 8.5; 5′-nucleotidases from other sources have likewise a pH optimum in this region.

Potency. The activity per milligram of the most highly purified preparations of seminal 5′-nucleotidase is about twice that of purified intestinal phosphatase. The concentration of the enzymic activity in seminal plasma is much higher than that of intestinal phosphatase in the intestinal mucosa.

[146] J. Reis, *Bull. soc. chim. biol.* **22,** 36 (1934).

[147] J. Reis, *Enzymologia* **2,** 183 (1937).

[148] J. Reis, *Enzymologia* **5,** 251 (1938).

[149] T. Mann, *Biochem. J.* **39,** 451 (1945).

[150] M. Wachstein and E. Meisel, *Science* **115,** 652 (1952).

[151] A. G. E. Pearse and J. L. Reis, *Biochem. J.* **50,** 534 (1952).

[152] A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.* **186,** 557 (1950).

[153] L. A. Heppel and R. J. Hilmoe, *J. Biol. Chem.* **188,** 665 (1951).

Specificity. The specificity of bull semen phosphatase was thoroughl investigated by Heppel and Hilmoe, who found that 5′-adenylic acid, 5′ uridylic acid, and nicotinamide-5-nucleotide, as well as ribose-5-phosphate were rapidly hydrolyzed by the enzyme, whereas all other phosphoric aci esters tested, in particular, the 2′- and 3′-ribonucleotides and the adeny pyrophosphates, were resistant toward the enzyme. Of particular interes is the fact that ribose-5-phosphate is hydrolyzed at a very considerable rat whereas glucose-6-phosphate, phosphogluconate, and 1,6-fructose diphosphate are practically resistant against the enzyme.

Michaelis-Menten constants. The affinity of seminal 5′-nucleotidase fo the purine and pyrimidine-5′-nucleotides ($K_{M\text{adenylic}} < 10^{-4}$, $K_{M\text{uridyli}}$ $< 10^{-4}$, $K_{M\text{cytidylic}} = 2 \times 10^{-4}$) is much higher than those for ribose-5′ phosphate ($K_{M\text{ribose-5-phosphate}} = 2.6 \times 10^{-3}$; $K_{M\text{nicotinamide-5-nucleotide}} = 1.$ $\times 10^{-3}$).

Activators and inhibitors. Magnesium ions in approximately 0.01 *M* concentration have considerable activating effect; they cannot be replaced b manganese ions. The extent of the magnesium activation depends on th nature of the buffer: Heppel and Hilmoe found that in tris(hydroxymethyl aminomethane buffer the enzyme has 75% of its full activity without th addition of magnesium ions, whereas, in glycine buffer the activity withou magnesium ions amounts only to 30% of the optimal activity.[67]

Fluorides in 0.01 *M* concentration inhibit the enzyme activity by 70% in 0.1 *M* concentration completely. Borate in 0.08 *M* concentration inhibited the activity 85% in comparison with glycine buffer at the same pH.

Stability. Crude as well as purified preparations of seminal 5′-nucleotidas can be preserved in active form for a considerable time in the frozen state before or after lyophilization.

b. 3′-Nucleotidase (formerly b-nucleotidase)

Shuster and Kaplan[154] discovered the presence in germinating rye gras and germinating barley of a phosphatase which specifically catalyzes th hydrolysis of 3′-nucleotides to nucleosides and inorganic phosphate. Enzymes of similar specificity occur in other vegetable materials, such as whea and corn leaves, soy bean leaves, and lawn grass leaves. Takadiastase likewise dephosphorylates 3′-nucleotides much faster than 2′- or 5′-nucleotides So far, the most powerful 3′-nucleotidase activity was found in germinatin rye grass. 2′-Nucleotides, 5′-nucleotides, and pyrophosphates were no hydrolyzed by purified preparations of the enzyme. The fact that one o the phosphoryl groups of coenzyme A is hydrolyzed by 3′-nucleotidase i interpreted by assigning to this phosphoryl group the 3′-position of th adenylic acid group. The phosphomonoester group of TPN, on the other hand, is resistant toward 3′-nucleotidase, and it is assumed that this group is esterified at the 2′-position of the adenylic acid moiety. The glycerophosphates and the hexose and pentose phosphates are not hydrolyzed by the enzyme.

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[154] L. Shuster and N. O. Kaplan, *J. Biol. Chem.* **201,** 535 (1953).

The phosphoric acid diester, diphenyl phosphate, is resistant to 3′-nucleotidase, but the phosphodiester linkages of ribopolynucleotides are hydrolyzed by the enzyme. During this hydrolysis, only up to 6% of the total phosphorus is liberated in the form of inorganic phosphate. This would suggest that the interlinkages of the ribopolynucleotides are cleaved at the 3′-phosphoric acid ester bonds. It cannot yet be decided whether the ribonuclease activity of 3′-nucleotidase preparations is a property of the 3′-nucleotidase itself, or whether it must be attributed to the presence of a second enzyme in the preparations.

pH Optimum. 3′-Nucleotidase has a rather narrow range of optimal activity in the neighborhood of pH 7.5.

Kinetics. Different 3′-nucleotides are hydrolyzed by 3′-nucleotidase at very different rates. Shuster and Kaplan found the following velocities: 3′-adenylic acid, 1; 3′-inosinic acid, 0.47; 3′-guanylic acid, 0.27; 3′-uridylic acid, 0.19; 3′-cytidylic acid, 0.11. The K_M values for some nucleotides are as follows: 3′-adenylic acid, 0.3×10^{-3} M; 3′-cytidylic acid, 2.5×10^{-3} M; 3′-uridylic acid, 2.7×10^{-3} M.

No additive effect on the hydrolysis rates were observed in mixtures of various 3′-nucleotides; this observation suggests the assumption that the hydrolysis of the various 3′-nucleotides is catalyzed by one enzyme.

The action of 3′-nucleotidase on 3′-nucleotides is competitively inhibited by 2′- and by 5′-nucleotides.

Inhibition of 50% of the optimal activity is effected by the following compounds: 5.9×10^{-3} M phosphate; 17×10^{-3} M arsenate; 0.9×10^{-3} M cysteine; 5.2×10^{-3} M potassium cyanide; 5×10^{-3} M glutathione. The effect of cysteine can be reversed by dialysis.

Heat Lability. Most of the enzyme activity is destroyed within 10 minutes at 80°, and within one or 2 minutes at 100°.

3. Phosphatases as Phosphotransferases between Phosphoric Acid Esters and Nucleosides

Brawerman and Chargaff[154a-c] obtained evidence for the power of various phosphatases to transfer phosphate groups from "low energy" donors, such as 5′-nucleotides or phenyl phosphates, to nucleosides. This transferase

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[154a] G. Brawerman and E. Chargaff, *J. Am. Chem. Soc.* **75,** 2020 (1953).

[154b] G. Brawerman and E. Chargaff, *J. Am. Chem. Soc.* **75,** 4113 (1953).

[154c] G. Brawerman and E. Chargaff, *Federation Proc.* **13,** 186 (1954); *Biochim. et Biophys. Acta* (in press).

activity is of interest as a mechanism for the biosynthesis of nucleotide The donor requirements are different for different phosphatases. 5′-Nucleotides are particularly efficient donors with malt phosphatase as the transfe enzyme, but ineffective with prostate phosphatase.[154b] On the other hand phenyl phosphate is a good phosphoryl donor with prostate phosphatase No essential differences between the efficiency of various donors were foun for liver phosphatase.[154c]

The structure of the nucleotides formed was different according to th nature of the transferring phosphatases. With the barley phosphatase, onl 5′-nucleotides were obtained (e.g., 5′-cytidylic, 5′-thymidylic acids) whereas with prostate phosphatase, all three isomers of each nucleotid were obtained. Between 5 and 10% of the added nucleosides (in 40 m concentrations) were phosphorylated at donor concentrations of approximately 200 mM.

IV. Nucleoside Kinases

Adenosine kinase was discovered in yeast by Ostern, Baranowski, and Terszakoweć,[154d] in 1938 and partially purified by Caputto[154e] and by Kornberg and Pricer[154f] in 1951, who demonstrated also the presence of a different kinase in some animal tissues. It catalyzes the reaction:

$$\text{adenosine} + \text{ATP} = \text{AMP} + \text{ADP}$$

pH Optima. The pH optimum of the yeast enzyme is between 6.9 and 7; that of the liver enzyme lies between 4.9 and 5.0.

Specificity. The yeast enzyme, as well as the liver enzyme, is specific for adenosine and for 2,6-diaminopurine riboside.[154f] No phosphorylation of guanosine, inosine, uridine, or cytidine could be detected.

Activators. The yeast enzyme is optimally activated by 0.02 M magnesium ions or by 0.002 M manganese ions. The enzyme can be stabilized by reduced glutathione or by an unknown stabilizing factor present in boiled yeast extracts.

Assay. According to Ostern *et al.*[154d] acetone-dried yeast is sufficiently active to permit the isolation of 5′-adenylic acid in good yield in large-scale experiments with adenosine as substrate.

In maceration juice or extracts of animal tissues, the enzyme can only be detected after a preceding purification designed to remove contaminating enzymes. Caputto used in his assay procedures manometric determinations of 5′-adenylic deaminase, phosphate balances, or chromatography, and he succeeded in the quantitative analytical separation of adenosine and the nucleotides by precipitation of the latter with zinc sulfate and barium hydroxide.

[154d] P. Ostern, T. Baranowski, and J. Terszakoweć, *Z. physiol. Chem.* **251**, 258 (1938).

[154e] R. Caputto, *J. Biol. Chem.* **189**, 801 (1951).

[154f] A. Kornberg and W. E. Pricer, *J. Biol. Chem.* **193**, 481 (1951).

The presence in yeast autolysates and pigeon liver extracts of a phosphokinase converting the 4-amino-5-imidazolecarboxamide riboside to its ribotide was reported by Greenberg.[154g]

V. Enzymes Acting on the Amino Groups of Purine and Pyrimidine Compounds

1. Enzymic Deamination of the Adenine Group

a. Behavior of Free Adenine

The tissues of many higher animals do not seem to contain enzymes capable of deaminating free adenine. Some statements to the contrary in the literature are based on indirect evidence such as the decoloration of methylene blue by adenine in the presence of xanthine dehydrogenase. It is now known that the enzymic dehydrogenation of adenine results in the formation of 2,8-dihydroxyadenine, and that it is not preceded by its deamination to hypoxanthine.[155,156] The metabolic resistance of the amino group of adenine in rats and dogs is demonstrated by Nicolaier's[155] observation that ingested adenine is transformed into 2,8-dihydroxyadenine, which forms crystalline deposits in the kidney. The mechanism of this transformation has recently been studied by Bendich, Brown, Philips, and Thiersch[156] who found that ingested 2-hydroxyadenine (isoguanine) and 8-hydroxyadenine can be converted to 2,8-dihydroxyadenine (see also Chapter 25). It should be pointed out, however, that the basis of comparative biochemical studies of this question is not broad enough to permit the conclusion that the resistance of the amino group of free adenine is representative of the behavior of this base in higher animals.

The desirability of extensive comparative studies is emphasized by the observations of Duchateau-Bosson and his collaborators,[157-159] who found that extracts of certain lower animals such as anodonta, crustaceans, and insects contain adenine deaminases, but lack enzymes capable of deaminating adenosine or adenylic acid. The authors consider the different distribution of the deaminases of the adenine group in lower and higher animals as an example of phylogenetic changes in the pattern of certain metabol pathways.

[154g] G. R. Greenberg, *Federation Proc.* **12,** 211 (1953).

[155] A. Nicolaier, *Z. klin. Med.* **45,** 359 (1902).

[156] A. Bendich, G. B. Brown, F. S. Philips, and J. B. Thiersch, *J. Biol. Chem.* **183,** 267 (1950).

[157] G. Duchateau-Bosson, M. Florkin, and G. Frappez, *Compt. rend. soc. biol.* **133,** 433 (1940).

[158] G. Duchateau-Bosson, M. Florkin, and G. Frappez, *Compt. rend. soc. biol.* **133,** 274 (1940).

[159] G. Duchateau-Bosson, M. Florkin, and G. Frappez, *Acad. roy. Belg.* **27,** 169 (1941).

b. *Adenyl Deaminase of Aspergillus Oryzae*

Mitchell and McElroy found in 1946 that takadiastase contains an en zyme capable of deaminating adenosine.[160] They succeeded in separatin taka-adenosine deaminase from some contaminating enzymes on a chro matopile in quantities suitable for isolation experiments.[161] Kaplan, Colo wick, and Ciotti[162] achieved a considerable further purification of the en zyme by acetone–alcohol and ammonium sulfate fractionations with th practically complete removal of phosphatases. According to Kaplan *et al.*[1 adenosine, adenosine-5′-phosphate, adenosine-3′-phosphate, ATP, ADP oxidized and reduced DPN, and adenosine-diphosphate-ribose are deami nated by the purified enzyme at rates decreasing in this order. Adenine adenosine-2′-phosphate and TPN are not deaminated.

The lack of specificity of the *Aspergillus oryzae* adenyl deaminase is i remarkable contrast to the highly selective action of animal adenyl de aminases. The deamination of DPN with preservation of the pyrophosphat bond represents the first example of the enzymic deamination of an adeny compound of a pyrophosphoryl dinucleotide.

pH Optimum. Aspergillus oryzae deaminase has a broad zone of optima activity between pH 5 and pH 8.

Michaelis-Menten Constants. The following K values were calculated fo the various substrates by Kaplan *et al.*:[162] adenosine, $0.6 \times 10^{-3}\ M$; ADP $0.7 \times 10^{-3}\ M$; 5′-AMP $0.8 \times 10^{-3}\ M$; ATP, $1.2 \times 10^{-3}\ M$; adenosine diphosphate-ribose $1.5 \times 10^{-3}\ M$; 3′-AMP, $1.7 \times 10^{-3}\ M$.

c. *Adenosine Deaminase*

Most tissues (intestines, liver, kidney, spleen, brain, striated muscle heart) of higher animals contain adenosine deaminase in very active con centrations. The enzyme was discovered by György and Röthler;[163] th high degree of its specificity was recognized by Schmidt[164] in an investiga tion on muscle deaminases, and its distribution in animal tissues was de scribed by Conway and Cooke.[165] Procedures for its partial purificatio from aqueous extracts of intestinal mucosa have been described by Brady[166] and by Kalckar.[167]

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[160] H. K. Mitchell and W. D. McElroy, *Arch. Biochem.* **10,** 351 (1946).

[161] H. K. Mitchell, M. Gordon, and F. A. Haskins, *J. Biol. Chem.* **180,** 1071 (1949).

[162] N. O. Kaplan, S. P. Colowick, and M. M. Ciotti, *J. Biol. Chem.* **194,** 579 (1952)

[163] P. György and H. Röthler, *Biochem. Z.* **187,** 194 (1927).

[164] G. Schmidt, *Z. physiol. Chem.* **179,** 243 (1928).

[165] E. J. Conway and R. Cooke, *Biochem. J.* **33,** 479 (1939).

Specificity. Adenosine deaminase is strictly specific toward adenine ribo- and deoxyribonucleosides,[165] and it is probable that both nucleosides are deaminated by the same enzyme. The amino groups of free adenine and of other purines, pyrimidines, and their derivatives, as well as those of the adenine nucleotides, are resistant to the enzyme. Byrne[167a] reported, however, that highly purified samples of adenosine deaminase from beef spleen were capable of hydrolysing the amino groups of 2′-adenylic acid and 2′, 3′,-cyclic adenylic acid as rates corresponding approximately to $\frac{1}{30}$ of the rate of the deamination of adenosine. The pH-optimum for the nucleotides was at pH 5.2, that for the nucleoside between pH 7.5 and 9.3. 3′- and 5′-Adenylic acid, DPN, and TPN were not appreciably deaminated.

pH Optimum. According to Kalckar, the pH optimum of adenosine deaminase is near the neutral point, but its activity is still considerable at pH 9 and pH 6.

Stability. Adenosine deaminase of intestinal mucosa is rapidly inactivated by standing at pH 3. On dialysis against water, the activity disappears rapidly and is not restored by combination with the dialysate.

Inhibitors. According to Brady,[166] the activity of intestinal adenosine deaminase is not influenced by low concentrations of fluoride, phosphate, and cyanide ions.

d. 5′-Adenylic Acid Deaminase

5′-Adenylic acid deaminase occurs in relatively high concentration in striated muscle[164] but is practically absent in heart.[165,168]

5′-Adenylic acid deaminase is a highly specific enzyme which converts 5′-riboadenylic acid to inosinic acid. According to Carter,[169] 5′-deoxyadenylic acid is slowly deaminated by the enzyme. All other amino compounds are resistant, in particular, the 5′-adenosine pyrophosphates and 2′- and 3′-adenosine phosphates. Owing to its high specificity, the enzyme is used as a convenient analytical tool for the quantitative determination of 5′-adenylic acid.

The enzyme can be easily prepared from muscle in enzymically homogeneous form. Its action can be followed either by ammonia determinations or more conveniently by measuring the changes of the absorption at 265 mμ caused by the conversion of the adenine to the hypoxanthine group.[167,170]

[166] T. Brady, *Biochem. J.* **36,** 478 (1942).

[167] H. M. Kalckar, *J. Biol. Chem.* **167,** 461 (1947).

[167a] W. L. Byrne, *Abstr. 126th Meeting Am. Chem. Soc., New York* p. 73C (1954).

[168] W. Kutscher and W. Sarreither, *Klin. Wochschr.* **26,** 605 (1948).

[169] C. E. Carter, *J. Am. Chem. Soc.* **73,** 1537 (1951).

[170] H. M. Kalckar, *J. Biol. Chem.* **167,** 429 (1947).

pH Optimum. 5′-Adenylic acid deaminase has a sharp optimum at pH 5.9.

Extractability. 5′-Adenylic deaminase is easily extracted from minced muscle by 2% solutions of sodium bicarbonate or, to a lesser extent, by water. Very little of the enzyme is extracted by 0.85% sodium chloride solution. Removal of soluble proteins by exhaustive extraction of minced rabbit muscle with the latter solution, and subsequent extraction of the residue with 2% sodium bicarbonate solution, yields a highly active deaminase extract, free of myoglobin, which represents a suitable starting material for further purification.

These solubility properties are probably responsible for the fact that purified myosin preparations frequently exhibit deaminating activity toward 5′-adenylic acid.[171] The association of this deaminase with myosin, however, is much looser than that of the Ca-activated adenosinetriphosphatase. At present, it is hardly justifiable to consider adenylic deaminase activity as an inherent property of myosin,[172] particularly in view of the absence of adenylic acid deaminase activity in heart muscle.

2. Enzymic Deamination of the Guanine Group: Guanase, Guanosine Deaminase, Guanylic Acid Deaminase

Unlike the adenine group, the guanine group can be deaminated enzymically by many tissues of higher animals in the form of the free base as well as in that of its riboside.[172a] Aqueous extracts of fresh rabbit liver or of acetone powders prepared from this tissue are capable of deaminating guanylic acid, guanosine, and guanine. The crude, as well as the purified, enzyme preparations used in these investigations contained, in addition, phosphatase, nucleoside phosphorylase, and possibly enzymes cleaving the purine-ribose linkage of nucleotides. The available evidence[173,174] does not permit a decision of the question as to whether or not guanosine and guanylic acid are deaminated before the cleavage of the guanine-ribose linkage.

Specificity of Guanase. Guanase is highly, but not absolutely, specific for guanine. According to Hitchings and Falco,[175] the enzyme also deaminates 1-methylguanine. Roush and Norris[176] found that highly purified guanase preparations prepared from rat liver according to Kalckar[167] deaminate azaguanine. The deamination of azaguanine is competitively inhibited by guanine. Measurable inhibitory effects of some pteridine derivatives (xanthopterine, 6-hydroxymethyl pteridine, and 6-formyl pteridine) on the enzymatic deamination of azaguanine were reported by Dietrich and Shapiro.[176a]

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[171] V. Sz. Hermann and G. Josepovits, *Nature* **164,** 865 (1949).

[172] B. A. Askonas, *Biochem. J.* **48,** 42 (1951).

[172a] For older references, see W. Jones, "Nucleic Acids," p. 70. Longmans Green & Co., London, 1920.

[173] G. Schmidt, *Z. physiol. Chem.* **208,** 185 (1932).

[174] Y. Wakabayashi, *J. Biochem. (Japan)* **28,** 185 (1938).

[175] G. H. Hitchings and E. A. Falco, *Proc. Natl. Acad. Sci. U. S.* **30,** 294 (1944).

[176] A. Roush and E. R. Norris, *Arch. Biochem.* **29,** 124 (1950).

pH Optima of Guanase. According to Roush and Norris, guanase deaminates guanine over a pH range between 5 and 9 with a rather flat optimum at pH 8; the deamination of azaguanine has a much sharper optimum at pH 6.5, and the activity decreases steeply at both sides of the optimum. At pH 8 the activity toward azaguanine is only slight.

Michaelis Constants. For Michaelis constants, Roush and Norris calculated for guanine as substrate $K_{M_{\text{guanine}}} = 5 \times 10^{-6}\ M$; for azaguanine, $K_{M_{\text{azaguanine}}} = 7 \times 10^{-5}\ M$ in phosphate buffers of pH 6.5. The high dilution of the substrates necessitated by their small solubilities, must be considered in the evaluation of these constants.

Assay Methods. Guanase may be assayed spectrophotometrically by determining the changes of the extinction at 245 mμ, or by determination of the liberated ammonia. The latter procedure requires substrate solutions of concentrations exceeding the solubility of guanine at the pH range of optimal activity. Sufficiently stable colloidal guanine suspensions can be prepared by neutralizing guanine solutions in dilute sodium hydroxide in the presence of 0.5% gelatin.[173]

3. Enzymes Deaminating the Cytosine Group

Very little information is available regarding deaminases of the cytosine group of nucleic acid derivatives. Highly active extracts of cytosine deaminase were obtained by Kream and Chargaff[177] from ground cells of yeast and *E. coli*. The extracts had no activity toward adenine and guanine. Cytidine and cytidylic acid (possibly after enzymic dephosphorylation) are deaminated by extracts of mouse kidney.[178]

4. Evidence for Transamination in the Purine and Pyrimidine Field

One of the least explored questions in nucleic acid metabolism is that of the formation of the amino groups of the nucleic acid purines and pyrimidines. Rapid turnover rates for the amino group of the adenine compounds of muscle were demonstrated by Kalckar and Rittenberg.[179] Only a few pertinent enzymic observations are so far available.

Weil-Malherbe[180] reported that the formation of ammonia during the incubation of dialyzed brain homogenates increased considerably over th sum of the amounts of ammonia in all controls when glutamine and inosin triphosphate were added to the system. The most plausible explanation o this observation is the assumption that the amide group of glutamine wa enzymically transferred to the hypoxanthine group of inosine triphosphate which was in turn deaminated by the enzymes of the homogenate.

[176a] L. S. Dietrich and D. M. Shapiro, *J. Biol. Chem.* **203,** 89 (1953).

[177] E. Chargaff and J. Kream, *J. Biol. Chem.* **175,** 993 (1948); J. Kream and E. Chargaff, *J. Am. Chem. Soc.* **74,** 4274, 5157 (1952).

[178] J. P. Greenstein, C. E. Carter, H. W. Chalkley, and F. M. Leuthardt, *J. Natl. Cancer Inst.* **7,** 9 (1946).

[179] H. M. Kalckar and D. Rittenberg, *J. Biol. Chem.* **170,** 455 (1947).

[180] H. Weil-Malherbe, *Biochem. J.* **54,** vi (1953).

Stephenson and Trim[180a] found that the rate of deamination of adenin by *E. coli* suspensions was strongly accelerated by catalytic amounts o adenosine. The authors discussed—very cautiously—the possibility of th deamination of the added adenosine to inosine and of the subsequent transamination of the amino group of adenine to inosine. It is just as reasonable however, to assume a phosphorolytic or nonphosphorolytic exchange of th whole adenine molecule with the hypoxanthine group of inosine.

According to Gunsalus and Tonzetich,[181] adenine, guanine, or cytosin are amino donors for glutamate formation from ketoglutaric acid by cell-free extracts of *E. coli*. Pyridoxal is required for the reaction; ammoniu ions are without effect.

VI. Enzymes Acting on the Linkages Between the Basic and the Carbohydrate Groups of Nucleic Acid Derivatives

The enzymic cleavage of the linkages between the basic and the carbohydrate groups of polynucleotide derivatives can take place either in th free nucleosides or in pyrophosphoryl dinucleotides (e.g., DPN). So far, n such cleavage has been observed on nucleic acids or polynucleotides.

The enzymic cleavage of *N*-glycoside bonds of nucleic acid derivative was first observed in nucleosides, and the term "nucleosidases" introduce by Levene and Medigreceanu[1] is still used.[182] In the current literature thi name tends to be replaced by the designations "nucleoside phosphorylases and "nucleoside hydrolases." Those terms are more satisfactory because o their adaptability to similar enzymes acting on nucleotides as substrates In the case of the latter enzymes, it might be advisable to change the term "nucleotide phosphorylases" (proposed by Saffran and Scarano[183]) to "nucleotide-1′-phosphorylases." Although the term "phosphorylases" still implied (in analogy to Cori's original designation) enzyme reaction of glycosidic groups with phosphate, this cannot be said for pyrophosphorylases In fact, most of the known nucleotide pyrophosphorylases act on group other than glycosidic groups.

The reversible phosphorolysis of nucleosides offers a plausible pathwa for the biosynthesis of nucleotides involving as its first step the biosynthesis of a nucleoside and as the second step its esterification with phosphate. The first experimental evidence for the occurrence of this second step in cells was reported by Ostern and Terszacoweć,[184] who found that acetone-dried yeast or toluene-poisoned yeast was capable of converting added adenosine to AMP and ATP in the presence of fructose diphosphate or phosphoglyceric acid.

[180a] M. Stephenson and A. R. Trim, *Biochem. J.* **32,** 1740 (1938).

[181] C. F. Gunsalus and T. Tonzetich, *Nature* **170,** 162 (1952).

[182] M. Dixon and R. Lemberg, *Biochem. J.* **28,** 2065 (1934).

[183] M. Saffran and E. Scarano, *Nature* **152,** 949 (1953).

Esterification of nucleosides with phosphate, however, is not the only, and perhaps not the most important, mechanism for the biosynthesis of nucleotides. Observations suggesting the cleavage of the bond between purine bases and ribose at the nucleotide stage had been made by Schmidt[173] in 1932 and by Wajzer and Baron[185] in 1949. Since 1952, several enzyme reactions have been found in which certain purines, pyrimidines, or some of their nitrogenous precursors are condensed with the 1′-carbon atoms of 5′-phosphorylated ribose derivatives to nucleotides. Hydrolases acting on the *N*-glucoside linkage of certain nucleotides have also been described. The study of these enzyme reactions which was initiated mainly in the laboratories of G. R. Greenberg,[186] of J. M. Buchanan,[187] of H. M. Kalckar,[188] and of A. Kornberg[189] is rapidly becoming one of the central problems of current research on nucleotide metabolism.

History. Purine nucleosidases were discovered by Levene and Medigreceanu[1] soon after the elucidation of the general structure of nucleotides. The specificity of these enzymes toward purines and their inactivity toward nucleosides of pyrimidines and dihydropyrimidines was recognized in early investigations by Levene.[190,191] Much later, Deutsch and Laser[192] discovered in Thannhauser's laboratory the presence of specific pyrimidine nucleosidases in bone marrow. An important advance was made when Klein[193] found in 1935 that purine nucleosidase prepared from beef spleen lost its activity by dialysis but was reactivated by phosphate or arsenate. The explanation for this behavior was given in 1947 by Kalckar,[194] who established the phosphorolytic nature of phosphate-dependent nucleosidases. Carter,[195] however, furnished the first evidence for the conclusion that not

[184] P. Ostern and J. Terszacoweć, *Z. physiol. Chem.* **250,** 155 (1937).

[185] J. Wajzer and F. Baron, *Bull. soc. chim. biol.* **31,** 750 (1949).

[186] G. R. Greenberg, *Federation Proc.* **13,** 745 (1954).

[187] W. J. Williams and J. M. Buchanan, *J. Biol. Chem.* **203,** 583 (1953).

[188] H. M. Kalckar, *Biochim. et Biophys. Acta* **12,** 250 (1953).

[189] A. Kornberg, I. Lieberman, and E. S. Simms, *J. Am. Chem. Soc.* **76,** 2027 (1954).

[190] P. A. Levene and I. Weber, *J. Biol. Chem.* **60,** 707 (1924).

[191] P. A. Levene, Yamagawa, and I. Weber, *J. Biol. Chem.* **60,** 693 (1924).

[192] W. Deutsch and R. Laser, *Z. physiol. Chem.* **186,** 1 (1927).

[193] W. Klein, *Z. physiol. Chem.* **231,** 125 (1935).

[194] H. M. Kalckar, *J. Biol. Chem.* **167,** 461 (1947).

[195] C. E. Carter, *J. Am. Chem. Soc.* **73,** 1508 (1951).

all nucleosidases are phosphorylases, but that hydrolytic enzymes for the cleavage of the bonds between purine or pyrimidine bases and ribose do exist. The cleavage of nucleosides is, therefore, another example of the existence of dual mechanisms for the enzymic cleavage of glycosidic bonds.

1. Nucleoside Phosphorylases

a. Purine Nucleoside Phosphorylases

(1) *Animal Enzymes.* The first thorough studies of these enzymes were carried out on purified rat and calf liver extracts by Kalckar,[194] who obtained active preparations from the supernatant solutions obtained by high-speed centrifugation (16,000 r.p.m.). The extracts were purified by ammonium sulfate fractionation. The fraction which precipitated between 0.4 and 0.6 saturation was further purified by isoelectric precipitation at pH 6. The enzyme activity was recovered in the supernatant.

An enzyme of very similar specificity was purified by Heppel and Hilmoe[196] from yeast autolysates by ammonium sulfate fractionation and subsequent adsorption on aged calcium phosphate gel. A 19-fold purification was achieved but considerable losses occurred during the procedure.

Specificity: liver enzyme. Originally Kalckar found that only ribo- and deoxyriboguanosine and -hypoxanthine were split by purine nucleoside phosphorylase of rat liver, and that adenosine, xanthosine, and the pyrimidine nucleosides were resistant. Cardini and his associates[197] as well as Friedkin[198,199] found later, however, that uridine and xanthosine were also split by highly concentrated liver enzyme. The phosphorolysis of uridine by highly concentrated solutions of purine nucleoside phosphorylase is most likely caused by contamination with thymidine phosphorylase (see below). Isoguanosine and adenine thiomethylriboside are not split by the enzyme.[200]

Highly purified purine nucleoside phosphorylase was recently obtained by Korn and Buchanan[201,202] from aqueous extracts of acetone-dried beef liver, by alcohol and ammonium sulfate fractionation and by adsorption on silica gel. The final preparation, which was approximately 200 times as active as the crude aqueous extract, was capable of converting adenine to inosine in the presence of ribose-1-phosphate.[202] It is as yet undecided whether the resistance of adenine toward the cruder enzyme preparation from rat liver is a peculiarity of this species or whether it is caused by the presence of an inhibitor in these enzyme preparations. Rowen and Kornberg[203] found that liver contains a nicotinamide nucleoside phosphorylase and reported evidence in favor of its identity with the purine nucleoside phosphorylase. Reduced nicotinamide riboside is not cleaved by the enzyme.[204]

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[196] L. A. Heppel and R. J. Hilmoe, *J. Biol. Chem.* **198,** 683 (1952).

[197] C. E. Cardini, A. C. Paladini, R. Caputto, and L. F. Leloir, *Acta Physiol. Latinoamer.* **1,** 57 (1950).

[198] M. Friedkin, *J. Am. Chem. Soc.* **74,** 112 (1952).

[199] M. Friedkin, *Federation Proc.* **11,** 216 (1952).

[200] M. L. Schaedel, M. J. Waldvogel, and F. Schlenk, *J. Biol. Chem.* **171,** 135 (1947).

[201] E. D. Korn and J. M. Buchanan, *Federation Proc.* **12,** 233 (1953).

[202] E. D. Korn, F. C. Charalampous, and J. M. Buchanan, *J. Am. Chem. Soc.* **75,** 3610 (1953).

(2) *Enzymes of Microorganisms*. According to Heppel and Hilmoe,[196] the yeast enzyme splits only guanosine, hypoxanthosine, and nicotinamide nucleoside, but is without action toward adenosine, xanthosine, and some synthetic purine nucleosides. No information on the behavior of the deoxyribonucleosides toward the yeast enzyme is available as yet. *E. coli* (strain 15, American Type Culture Collection No. 9723) contains potent purine and pyrimidine deoxynucleoside phosphorylases according to Manson and Lampen.[205]

Mechanism of the Reaction (see also Chapter 24). With hypoxanthosine as substrate, the following reaction is catalyzed by the purine nucleoside phosphorylase:[194]

$$\text{hypoxanthosine} + \text{phosphate} \rightleftharpoons \text{hypoxanthine} + \text{ribose-1-phosphate}$$

The phosphate in this reaction can be replaced by arsenate; Heppel and Hilmoe state that the rate of arsenolysis of hypoxanthosine is approximately two-thirds of that of its phosphorolysis. 1-Riboarsenate undergoes spontaneous hydrolysis according to Manson and Lampen;[206] this observation probably accounts for Klein's opinion (based on the results of a reductometric assay procedure) that arsenate would be a more effective activator of nucleosidase than phosphate. It follows from the equation that, for example, a mixture of guanine riboside, hypoxanthine, and phosphate is transformed by the enzyme into a mixture of guanine and hypoxanthine ribosides and ribose-1-phosphate. This has been verified by Friedkin and Kalckar[207] for the deoxyribosides.

According to Friedkin and Kalckar, a mixture of ribo- and deoxyribonucleosides is not phosphorylated at a higher rate than equimolar amounts of the pure nucleosides. This suggests that the phosphorolysis of purine ribo- and deoxyribonucleosides is catalyzed by the same enzyme.

Influence of pH. The optimum for the action of the yeast enzyme was found near pH 7 by Heppel and Hilmoe;[196] the experiments with the liver

[203] J. W. Rowen and A. Kornberg, *J. Biol. Chem.* **193,** 497 (1951).
[204] H. M. Kalckar, *Biochim. et Biophys. Acta* **12,** 250 (1953).
[205] L. A. Manson and J. O. Lampen, *J. Biol. Chem.* **193,** 539 (1951).
[206] L. A. Manson and J. O. Lampen, *J. Biol. Chem.* **191,** 95 (1951).
[207] M. Friedkin and H. M. Kalckar, *J. Biol. Chem.* **184,** 437 (1950).

enzyme were carried out at pH 7.5, but no systematic pH activity curves have been reported.

Influence of Substrate Concentration. The Michaelis-Menten constants o the calf liver enzyme are 1.7×10^{-5} M for ribonucleosides, 1.8×10^{-5} M for deoxyribonucleosides, and 1.1×10^{-3} M for nicotinamide nucleoside According to Heppel and Hilmoe,[196] the yeast enzyme has the higher K_M values of 1.1×10^{-3} M for guanosine, 6.5×10^{-4} M for nicotinamide riboside, and 8×10^{-4} M for phosphate.

Equilibrium Constant. The equilibrium of the phosphorolysis of purine nucleosides is in favor of nucleoside formation. The equilibrium constant for hypoxanthosine phosphorolysis at pH 7.4 is 0.03 according to determinations with the yeast enzyme. The value for nicotinamide nucleoside phosphorolysis in which H^+ enters is 1×10^{-3}.[203,208]

Stability. Highly purified liver enzyme preparations can be stored at −20° with very little loss, whereas the yeast nucleoside phosphorylase is less stable, at least in highly purified conditions.

Assay Methods. In the earlier investigations of the period preceding the work of Kalckar,[194] the activity of nucleosidases was assayed reductometrically. The liberation of reducing groups after the action of nucleoside phosphorylases is, however, a secondary reaction caused by acid hydrolysis of the highly acid-labile ribose-1-phosphate.

In recent investigations, the action of the enzyme was measured either by determination of inorganic phosphate or of the liberated purines according to the sensitive and convenient procedures developed by Kalckar.[194] The formation of deoxyribosides can also be followed with the microbiological method of Hoff-Jørgensen[209,210] which is based on the observation that *Thermobacterium acidophilus* R26 (Orla Jensen Collection) requires deoxyribosides for growth.

b. *Pyrimidine Nucleoside Phosphorylases*

The first observations suggesting the existence of specific phosphorylases for pyrimidine nucleosides were made by Deutsch and Laser,[192] who discovered the power of bone marrow extracts to cleave deoxyribonucleosides of pyrimidines, and by Klein,[193] who demonstrated the phosphate—or arsenate—requirement of these enzymes. He also found that kidney extracts were much more active toward uridine than toward deoxyriboguanine, whereas the opposite behavior was found for spleen extracts. In Klein's experiments guanine "inhibited" the cleavage of purine nucleosides, but not that of uridine. The cleavage of cytidine was much slower than that of uridine and thymidine.

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[208] L. J. Zatman, N. O. Kaplan, and S. P. Colowick, *J. Biol. Chem.* **200,** 197 (1953).

[209] E. Hoff-Jørgensen, *J. Biol. Chem.* **178,** 525 (1949).

[210] E. Hoff-Jørgensen, M. Friedkin, and H. M. Kalckar, *J. Biol. Chem.* **184,** 461 (1950).

Paege and Schlenk[211] demonstrated the presence of potent uracil ribonucleoside phosphorylases in aqueous cell-free extracts of *E. coli*, *Aerobacter aerogenes*, and *Micrococcus lysodeicticus*. Cytidine was inert toward these enzymes.

Direct evidence for the existence of specific pyrimidine nucleoside phosphorylases was subsequently furnished by the purification of two enzyme fractions whose properties will be briefly described below.

(1) *Animal Thymidine Phosphorylase*. The reversible phosphorolytic cleavage of deoxythymidine was first observed in kidney extracts by Manson and Lampen.[211a,b] Friedkin[211c,d] obtained from horse liver a highly purified thymidine nucleoside phosphorylase.

Specificity. Friedkin's enzyme catalyzes the reversible exchange of the phosphoryl group of deoxyribose-1-phosphate and—at slower rates—of ribose-1-phosphate with thymine or uracil. 2-Thiouracil, 5-aminouracil, 5-iodouracil, and 5-bromouracil are likewise substrates of the enzyme. 2-Deoxythymidine was isolated by Friedkin and Roberts;[211c,d] thiouracil riboside and deoxyriboside were isolated by Strominger and Friedkin[211e] from digests of the respective substrate mixtures with thymidine phosphorylase.

Cytosine and orotic acid, as well as adenosine, guanosine, and hypoxanthosine, are inert in the enzyme system.

Equilibrium. Since thymine inhibits the reaction by an unknown mechanism, a well-defined equilibrium constant has not been obtained. At sufficiently high concentrations of deoxyribose-1-phosphate 80 to 90% thymine formation was observed.

pH optimum. The pH optimum for the enzymic arsenolysis catalyzed by thymidine phosphorylase is between 5.7 and 6. The activity decreases rapidly at the acid, slowly at the alkaline, side of the optimum and has a plateau at 63% of the optimal activity between pH 8.1 and 8.8.

Heat inactivation. The activity decreases by 9% during 8 minutes' heating at 50°, by 44% at 60°, and by 97% at 70°.

(2) *Bacterial Uridine Phosphorylase*. A specific uridine phosphorylase was purified from *E. coli* E-26 (Iowa State College Laboratory culture) by Paege and Schlenk[211f] by ammonium sulfate fractionation, adsorption on aluminum hydroxide, and elution with sodium phosphate.

Specificity. The enzyme catalyzes only the reversible phosphorolysis of uracil riboside. Adenosine, guanosine, hypoxanthine, ribocytidine, and deoxythymidine are inert toward the enzyme.

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[211] L. M. Paege and F. Schlenk, *Arch. Biochem.* **28,** 348 (1950).

[211a] L. A. Manson and J. O. Lampen, *Federation Proc.* **8,** 224 (1949).

[211b] L. A. Manson, Thesis, Washington University, St. Louis, 1949.

[211c] M. Friedkin and D. Roberts, *J. Biol. Chem.* **207,** 245 (1954).

[211d] M. Friedkin and D. Roberts, *J. Biol. Chem.* **207,** 257 (1954).

[211e] D. B. Strominger and M. Friedkin, *J. Biol. Chem.* **208,** 663 (1954).

[211f] L. M. Paege and F. Schlenk, *Arch. Biochem. and Biophys.* **40,** 57 (1952).

Equilibrium constant. The equilibrium constant of 0.62 is less in favor of the synthetic direction than that of purine nucleoside phosphorylases.

pH Optimum. The optimal pH is near pH 7.2.

Assay. The activity of the enzyme can be measured either by colorimetric pentose determination (pyrimidine ribosides yield only very little pigment in comparison with equimolar amounts of ribose-1-phosphate or free ribose) or by ultraviolet spectrophotometry.

2. NUCLEOSIDE HYDROLASES

a. Purine Nucleoside Hydrolase of Yeast

Heppel and Hilmoe[196] found in 1952 that autolysates of bakers' yeast were capable of splitting nucleosides in absence of phosphate or arsenate. They succeeded in separating the nucleoside hydrolase from the nucleoside phosphorylase by ammonium sulfate fractionation and in achieving further purification of the hydrolase by subsequent adsorption on calcium phosphate gel. The presence of a purine nucleoside hydrolase in potatoes was likewise demonstrated by Heppel and Hilmoe.

Specificity. The purified nucleoside hydrolase splits adenosine, guanosine, hypoxanthine, xanthosine, nicotinamide nucleoside, and some synthetic nucleosides such as 2,6-diaminopurine-9-ribofuranoside. Amongst the latter are D-ribopyranosyl adenine, D-glucopyranosyl adenine, D-arabofuranosyl adenine, D-ribofuranosyl adenine, which were substituted at the 2-carbon, and D-ribofuranosyl nucleosides of some synthetic purines.

Mechanism. The enzyme catalyzes the reaction:

$$\text{hypoxanthosine} + \text{water} = \text{D-ribose} + \text{hypoxanthine}$$

No exchange reaction was detected in the presence of isotopically labelled free purine in the reaction mixture.

The pH optimum of the purine nucleoside hydrolase is somewhat higher than that of the phosphorylase, namely, at about pH 8.

b. Uridine Hydrolase of Yeast

Carter[195] obtained from autolysates of bakers' yeast in 1950 a highly specific uridine nucleosidase which did not require phosphate or arsenate for its activity. No hydrolytic uridine nucleosidase has been found so far in mammalian tissues. The enzyme was partially purified 10 to 15 times by ammonium sulfate fractionation. Adenine, inosine, guanosine, cytidine, and thymidine were not degraded by the enzyme. The reaction followed a unimolecular course up to 85% of hydrolysis. The pH optimum was found to be at pH 7.

Activity Determination. The activity of the hydrolytic uridine nucleosidase can be determined spectrophotometrically by utilizing the facts that, at pH 7, the molar extinction of uridine at 280 mμ is 3.5×10^3, that of uracil 1.4×10^3, or that in 0.01 *N* sodium hydroxide the molar extinctions of uridine and uracil are 30 and 5.4×10^3, respectively.

c. Nucleoside Hydrolases of Lactobacillus pentosus

Lampen and Wang[211g] demonstrated that extracts of *Lactobacillus pentosus* contain nucleoside hydrolases catalyzing the cleavage of purine ribosides (including uric acid riboside) and pyrimidine ribosides. The hydrolysis of the pyrimidine nucleosides was enhanced, that of the purine nucleosides depressed, by phosphate and arsenate ions. The authors demonstrated that the enhancing effects of phosphate and arsenate on the cleavage of the pyrimidine nucleosides were caused by the stabilizing effect of bivalent anions on the enzyme proteins, but not by the participation of these ions in the enzyme reaction. The hydrolysis of the purine ribosides was strongly inhibited by phosphate and arsenate ions.

3. NUCLEOTIDE-1′-PHOSPHORYLASE

The requirement of ribose-5-phosphate and of ATP for nucleotide synthesis in pigeon liver was first recognized by Williams and Buchanan.[187]

The role of ribose compounds esterified with phosphoryl groups in the 1 as well as in the 5 position as intermediaries in the enzymic formation of nucleotides was suggested by Saffran and Scarano[183] who found that a phosphorylated ribose believed to be 1,5-ribose diphosphate was transformed into 5′-adenylic acid by pigeon liver homogenates in presence of C^{14}-labeled adenine. It is very possible, however, that the actual intermediary in this and in similar reactions is 1-pyrophosphoryl, 5-phosphorylribose, which was isolated by Kornberg *et al.*[189] (see next paragraph). According to J. M. Buchanan (private communication to the author) this substance is the intermediary involved in the enzymic synthesis of inosinic acid in pigeon liver extracts in presence of ATP and ribose-5-phosphate.

4. NUCLEOTIDE-1′-PYROPHOSPHORYLASE

According to Kornberg *et al.*,[189] a partially purified enzyme fraction from yeast catalyzes the reversible condensation between adenine or orotic acid and a pyrophosphoryl ribose compound of the probable strucure of 1-pyrophosphoryl-ribose-5-phosphate to 5′-adenylic acid or 5′-orotidyl nucleotide, respectively. Detailed data regarding the equilibria and the kinetics of these important enzyme reactions are not as yet available. The phosphorylated ribose compound was obtained by the action of a purified enzyme preparation from pigeon liver acetone powder on a mixture of ATP and ribose-5-phosphate.

[211g] J. O. Lampen and T. P. Wang, *J. Biol. Chem.* **198,** 385 (1952).

Orotidyl nucleotide is decarboxylated by another enzyme fraction from yeast to uridylic acid. It appears that the enzymic decarboxylation of orotic acid requires its intermediate conversion to 5′-orotidylic acid.

The complete enzyme system is therefore capable of converting adenine or orotic acid in the presence of ribose-5-phosphate and ATP to 5′-adenylic or 5′-uridylic acid, respectively. The presence of the complete enzyme system in rat and pigeon liver has been demonstrated by Hurlbert and Reichard.[211h]

Karger and Carter[212] obtained evidence for the enzymic conversion of orotic acid to uracil by liver extracts and for the essential role of uridylic acid as an intermediary step in this conversion.

5. DPN Hydrolases

A more detailed description of the enzymes which hydrolyze DPN to nicotinamide and adenosine diphosphorylribose is beyond the scope of this book since they are not enzymes of nucleic acid metabolism in the strict sense. Two aspects, however, are of interest in correlation with the properties of the enzymes discussed in this chapter: (a) The group of DPN hydrolases is so far the only group of hydrolases known to cleave an *N*-glycoside-like linkage in a compound whose structure resembles that of dinucleotides. (b) Some DPN hydrolase preparations catalyze phosphate-independent exchange reactions of the bound nicotinamide group of DPN. With regard to these exchange reactions, the occurrence of three different types of DPN hydrolases has so far been established: (1) *DPN hydrolase of Neurospora crassa* was obtained as a soluble, highly purified preparation by Kaplan, Colowick, and Ciotti.[213] It is competitively inhibited by nicotinamide in relatively high concentration ($1.45 \times 10^{-1}\,M$ is required for 50% inhibition) and does not catalyze the exchange of the nicotinamide group of the substrate with free nicotinamide. (2) *DPN hydrolases of some animal organs such as beef spleen, lung, and brain.* The enzyme of beef spleen was obtained as a particulate centrifugal fraction, the specific activity of which was approximately 1% that of the purified Neurospora enzyme. It is inhibited to a degree of 50% by a $1.5 \times 10^{-3}\,M$ nicotinamide concentration. This inhibition is noncompetitive with respect to DPN, and must be attributed to the enzymically catalyzed exchange of the bound nicotinamide group with free nicotinamide. The exchange was demonstrated with C^{14}-labelled nicotinamide.[214] (3) Whereas the action of some of the animal DPN hydrolases is strongly inhibited by the antituberculous drug isonicotinic acid hydrazide, that of some other animal DPN hydrolase preparations (enzyme preparations from pigs' brain, human spleen and prostate) is not appreciably influenced by this substance.[215] Zatman *et al.* demonstrated that the latter enzyme preparations are capable of catalyzing the exchange of the nicotinamide group of DPN with isonicotinic acid hydrazide.

[211h] R. B. Hurlbert and P. Reichard, *Acta Chem. Scand.* **8**, 701 (1954).

[212] B. Karger and C. E. Carter, personal communication (1954).

[213] N. O. Kaplan, S. P. Colowick, and M. M. Ciotti, *J. Biol. Chem.* **194**, 579 (1952).

[214] L. J. Zatman, N. O. Kaplan, and S. P. Colowick, *J. Biol. Chem.* **200**, 197 (1953).

They isolated from incubation mixtures of the enzyme from pigs' brain with DPN and isonicotinic hydrazide a compound which appears to be an analogue of DPN in which the nicotinamide group is replaced by isonicotinic acid hydrazide. They therefore postulate as a working hypothesis the formation of a complex between the enzyme and the adenosine diphosphoribosyl group. This complex is believed to contain the free energy of the glycosidic bond. Water and nicotinamide or isonicotinic hydrazide are believed to compete for the reactions with this complex.

VII. Xanthine Oxidase

The discovery of xanthine oxidase dates back to Horbaczewski,[216] who observed in 1882 that extracts of various tissues were capable of oxidizing xanthine to uric acid. The early history of the enzymic formation and destruction of uric acid is connected with the work of many outstanding investigators at the end of the 19th century and cannot be reviewed in detail in this article. Much pertinent information can be found in the monograph on uric acid by McCrudden.[217] It is worth mentioning that as early as 1905, Schittenhelm[218] successfully applied fractionation with ammonium sulfate and dialysis to the partial purification of xanthine oxidase from spleen extracts. His preparation was a mixture of xanthine oxidase and guanase, but was free of uricase activity. The model for Schittenhelm's method was the earlier use of ammonium sulfate for enzyme fractionation by Jacoby,[219] who developed his procedure in 1899 in Hofmeister's laboratory. Morgan, Stewart, and Hopkins[220] found that milk contained a dehydrogenase which catalyzed the oxidation of hypoxanthine and xanthine to uric acid, and raised the question of its identity with the aldehyde dehydrogenase discovered earlier in milk by Schardinger.[221]

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[215] L. T. Zatman, N. O. Kaplan, S. P. Colowick, and M. M. Ciotti, *J. Am. Chem. Soc.* **75,** 3293 (1953).

[216] J. Horbaczewski, *Monatsh.* **12,** 221 (1882).

[217] F. H. McCrudden, "Uric Acid." Boston, 1905.

[218] A. Schittenhelm, *Z. physiol. Chem.* **43,** 228 (1904).

[219] M. Jacoby, *Z. physiol. Chem.* **30,** 135 (1900).

[220] E. J. Morgan, C. P. Stewart, and F. G. Hopkins, *Proc. Roy. Soc.* (*London*) **B94,** 109 (1922–1923).

[221] F. Schardinger, *Z. Untersuch. Nahr. u. Genussm.* **5,** 22 (1902).

Ball[222] and later Corran, Dewan, Gordon, and Green[223] succeeded in a considerable purification of the enzyme obtained from milk and established its nature as that of a conjugated protein containing a flavin adenine dinucleotide as a prosthetic group which can be separated from the protein by heat or treatment with acid or alcohol. The prosthetic group of xanthine oxidase can replace the coenzyme of amino acid oxidase in Warburg and Christian's[224] test, and both flavin adenine dinucleotides have closely similar absorption spectra.

Evidence suggesting the participation of the flavin group of xanthine oxidase in its catalytic activity was obtained by Ball, and his conclusions were later supported by Horecker and Heppel[225] and by Morell[226] on much purer enzyme preparations. Morell established the proportionality of the catalytic activity of his preparations with their contents of a specific flavin compound. This was inferred from the fact that only a part of the total flavine of xanthine oxidase is reduced by the substrate. According to observations by Mackler *et al.*,[226a] this behavior must be attributed to the oxido-reduction equilibria between the enzyme- and the substrate-systems. The standard potential of xanthine oxidase is more negative than those of other flavoproteins.

The essential role of the flavin adenine dinucleotide for the catalytic action of xanthine dehydrogenase is further supported by the striking parallelism between the riboflavin content of the diet and the xanthine oxidase concentration in the tissues of growing rats[227]—a parallelism which resembles the behavior of other respiratory enzymes.

Methylene blue,[220] oxygen,[222] or cytochrome c[225] can act as hydrogen acceptors.

In addition to flavine adenine dinucleotide, xanthine oxidase has another, possibly iron containing chromophoric group.[226a,227a] The presence of this group is the reason for the differences between the absorption spectrum of xanthine oxidase and that of a flavoprotein.

The complex nature of xanthine oxidase is impressively demonstrated by the surprising identification of sodium molybdate as an additional dietary factor required for maintaining normal xanthine oxidase levels in the livers and particularly in the intestines of weanling rats and some other mammals.[227,228] According to Westerfeld and his collaborators,[229] xanthine dehydrogenase preparations purified by Ball's method contained 0.03% molybdenum. It is possible that the molybdenum-containing group represents the "second active group" of xanthine oxidase already suspected by Ball.[222]

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[222] E. G. Ball, *J. Biol. Chem.* **128,** 51 (1939).

[223] H. S. Corran, J. G. Dewan, A. H. Gordon, and D. E. Green, *Biochem. J.* **33,** 1694 (1939).

[224] O. Warburg and W. Christian, *Biochem. Z.* **298,** 150 (1938).

[225] B. L. Horecker and L. A. Heppel, *J. Biol. Chem.* **178,** 683 (1949).

[226] D. B. Morell, *Biochem. J.*, **51,** 657 (1952).

[226a] B. Mackler, H. B. Mahler, and D. E. Green, *J. Biol. Chem.* **210,** 149 (1954).

[227] E. C. de Renzo, E. Kaleita, P. Heyther, J. J. Cleson, B. L. Hutchings, and J. H. Williams, *J. Am. Chem. Soc.* **75,** 753 (1953).

[227a] D. A. Richert and W. W. Westerfeld, *J. Biol. Chem.* **209,** 179 (1954).

The role of molybdenum as a functionally important component of xanthine oxidase was demonstrated by Totter,[229a] by Green and Beinert,[229b] and by Mackler *et al.*[226a] Totter *et al.* found that labeled molybdate injected into a cow appeared in the xanthine oxidase isolated from the milk, and that the proportion between the molybdenum and the flavine remained constant at the value of 0.5. Green and Beinert arrived at the same proportion in their analyses of highly purified preparations of the enzyme. According to Mackler *et al.*, molybdenum can be removed from the enzyme by dialysis in alkaline solution. The molybdenum-free enzyme catalyzes the oxidation of substrate by indophenol or oxygen, but is incapable of reacting with one-electron acceptors such as cytochrome c or ferric cyanide. On addition of molybdic oxide, this catalytic function is restored. This specific role of the metal in facilitating the reaction of the enzyme with one-electron acceptors applies to other enzymes belonging to the group of metalloflavoproteins.

The metal-free xanthine oxidase can be partially reactivated by uranyl ions, but not by tungstate or by any representative of a large number of other metals. The metal-catalyzed oxidation of the substrate by one-electron acceptors has an absolute requirement for phosphate ions.

The role of molybdenum is not an exceptional property of xanthine oxidase since the nitrate reductase of *Neurospora crassa* was shown by Nason and Evans[229c] to contain flavine adenine dinucleotide and molybdenum as essential constituents.

According to Corran *et al.*,[223] the xanthine oxidase of milk is most likely identical with xanthine oxidase of liver, but some observations suggest that the enzymes in milk and in tissues might be associated with different carrier proteins.

Assay Methods. Xanthine oxidase may be assayed by the reduction of methylene blue,[229,230] by the manometric determination of oxygen up-

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[228] D. A. Richert and W. W. Westerfeld, *J. Biol. Chem.* **203,** 915 (1953).

[229] W. W. Westerfeld, J. M. McKibbins, J. C. Roemel, and M. F. Hilfinger, *Am. J. Physiol.* **157,** 184 (1949).

[229a] J. R. Totter, W. T. Burnett, Jr., R. A. Monroe, I. B. Witney, and C. L. Comer, *Science* **118,** 555 (1953).

[229b] D. E. Green and H. Beinert, *Biochim. et Biophys. Acta* **11,** 599 (1953).

[229c] A. Nason and H. J. Evans, *J. Biol. Chem.* **202,** 655 (1953).

[230] V. H. Booth, *Biochem. J.* **32,** 494 (1938).

take,[222,231,231a] by the spectrophotometric determination of reduced cytochrome c, or by determination of the uric acid formed.

Specificity.[230] Xanthine oxidase catalyzes the oxidation of hypoxanthine, xanthine, adenine (to dihydroxyadenine),[232] 8-hydroxypurine, 6-amino-8-hydroxypurine, 6-amino-2-hydroxypurine, 2-azaadenine,[232a] and xanthopterin.[234] It is noteworthy that the oxidation of the aminopurines is not accompanied by the formation of ammonia.

Even highly purified preparations of xanthine oxidase are active toward a considerable number of aliphatic and aromatic aldehydes and toward DPNH.

Certain substances related to aldehydes such as chloral hydrate, butyrylchloraldehyde, paraldehyde, glucosone, glucose, mannose, and gluconate are not oxidized by the enzyme.

Inhibitors. Xanthine oxidase is completely and irreversibly inhibited by low sodium cyanide concentrations (e.g., 0.0025 M).[222,235,235a] The reaction of the cyanide with the enzyme protein takes an appreciable time and the cyanide effect can only be demonstrated if the solutions of the enzyme and of the cyanide are mixed before the addition of the substrate. Hypoxanthine and other purines, e.g., uric acid, protect the enzyme against the effect of cyanide, presumably because of their higher affinities to the enzyme. The fact that not only the oxidation of hypoxanthine and xanthine, but also that of aldehydes, is protected by purines against the effect of cyanide strongly suggests the identity of xanthine oxidase and the Schardinger enzyme. Xanthine oxidase is inhibited by borate ions, most likely owing to the formation of borate complexes with the ribityl group of the riboflavin moiety of the enzyme. In experiments of Roush and Norris,[236] the inhibition which is competitive was 50% at concentrations of 0.03 M borate and 0.034 mM xanthine.

Effect of some substances of quinoid structure. Xanthine oxidase of liver is practically completely inhibited by oxidized *p*-aminophenol at 1×10^{-4} M.[236a] The inhibition can be fully reversed by reductive removal of the inhibitor from the enzymic digest. With the milk enzyme, the effects of

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[231] D. A. Richert, S. Edwards, and W. W. Westerfeld, *J. Biol. Chem.* **181,** 254 (1949).

[231a] S. B. Dhungat and A. Sreenivasan, *J. Biol. Chem.* **208,** 845 (1954).

[232] H. Klenow, *Biochem. J.* **50,** 404 (1952).

[232a] E. Shaw and D. W. Woolley, *J. Biol. Chem.* **194,** 641 (1952).

[233] H. Wieland and R. Liebig, *Ann.* **555,** 146 (1944).

[234] H. M. Kalckar, N. O. Kjeldgaard, and H. Klenow, *Biochim. et Biophys. Acta* **5,** 575 (1950).

[235] A. Szent-Györgyi, *Biochem. Z.* **173,** 275 (1926).

[235a] M. Dixon and D. Keilin, *Proc. Roy. Soc.* (*London*) **B119,** 159 (1936).

[236] A. Roush and E. R. Norris, *Arch. Biochem.* **29,** 344 (1950).

[236a] F. Bernheim and M. C. L. Bernheim, *J. Biol. Chem.* **123,** 307 (1938).

quinone imine are variable, presumably because of the presence of a substance which reacts with quinone imine. Xanthine oxidase of milk is inhibited to an extent of 50% by pyrogallol at 1×10^{-4} *M* concentration.[236b] Other known oxidases of liver are not appreciably inhibited by quinone imine. Many other phenols such as *p*-nitrophenol, *p*-bromophenol, phloroglucinol, anthraquinone, and α-naphthol do not significantly influence the action of xanthine oxidase.

Effect of pterin derivatives. Kalckar *et al.*[236c] found that 2-amino-4-hydroxy-6-formylpterin inhibits xanthine oxidase practically completely at a concentration of 10^{-4} *M*. 2-Amino-4-hydroxy-6-carboxypterine[236d] and 2-amino-4-hydroxy-6-hydroxymethylpterin[236d] inhibit the enzyme at higher concentrations. Other respiratory enzymes tested were not inhibited by these pterins.

It is of interest that these specific inhibitors of xanthine oxidase also have strong depressing effects on the endogenous oxygen consumption of liver homogenates. This raises the question of the role of xanthine oxidase in the endogenous respiration of isolated tissue preparations.

L-Ascorbic acid[236e] at 1×10^{-4} *M* inhibits irreversibly most of the activity of xanthine oxidase purified from cream.

Antabuse[236f] (tetraethylthiuram disulfide, $(C_2H_5)_2N(CS) \cdot S \cdot S \cdot (SO)N(C_2H_5)_2$, has a considerable inhibitory effect on crude and purified preparations of xanthine oxidase from rat liver, but not on preparations from milk. The inhibition of the liver enzyme can be overcome by methylene blue.

The differences in the behavior of xanthine oxidase preparations from milk and from tissues toward inhibitors are ascribed to the association of the enzyme with different carrier substances depending on the source of the preparations. An interesting example of such an association is furnished by the observation of Philpot,[236g] who found that the xanthine oxidase activity of milk is small until it was exposed to temperatures below 15°. The solidification of the fats releases the enzyme which was associated with the fat globules of the milk.

Copper ions inactivate xanthine oxidase in very small concentrations.[236h] The use of water distilled from copper vessels must therefore be avoided during experiments with this enzyme.

[236b] S. J. Gray and R. Z. Felsher, *Proc. Soc. Exptl. Biol. Med.* **59,** 287 (1947).

[236c] H. M. Kalckar, N. O. Kjeldgaard, and H. Klenow, *J. Biol. Chem.* **174,** 771 (1940).

[236d] H. G. Petering and J. A. Schmitt, *J. Am. Chem. Soc.* **72,** 2995 (1950).

[236e] P. Feigelson, *J. Biol. Chem.* **197,** 843 (1952).

[236f] D. A. Richert, R. Vanderlinde, and W. W. Westerfeld, *J. Biol. Chem.* **186,** 261 (1950).

[236g] F. J. Philpot, *Biochem. J.* **32,** 2013 (1938).

[236h] M. R. Stetten and C. L. Fox, Jr., *J. Biol. Chem.* **161,** 333 (1945).

VIII. Enzymes Involving the Opening of the Purine Ring

Up to the discovery of 4-amino-5-imidazolecarboxamide[236h,i] and it role in the formation of the purine ring, the only enzyme known to catalyz the biological degradation of the purine ring was uricase. Whereas the latte enzyme reaction is most likely irreversible in the sense that no evidenc exists for the participation of allantoin and its intermediary precursors i purine biosynthesis, the formation of 4-amino-5-imidazolecarboxamide compounds has been shown to be an important step in the biological formatio of the purine ring. The study of the enzymes involved in the metabolism o 4-amino-5-imidazolecarboxamide is still in the early stages and the role o this compound in purine biosynthesis is discussed in Chapter 23, but, i anticipation of the increasing importance of this field, a brief review of th available observations is included in this chapter.

In contrast to the action of uricase, the enzyme reactions involving th 4-amino-5-imidazolecarboxamide group occur exclusively on nucleotid compounds.

1. INOSINIC ACID TRANSFORMYLASE

An enzyme fraction obtained from pigeon liver extracts by fractional precipitation with alcohol catalyzes the reversible[236j,k] transformation of inosinic acid to 4-amino-5-carboxamide ribotide in the presence of certain formyl acceptors. The yield of the reaction product is strongly dependent on the concentration of inosinic acid. The reaction requires ATP and magnesium ions.[236l,m] It is enhanced by glycine (but not by other amino acids such as L-methionine, L-alanine, L-serine, L-leucine, and sarcosine) and by leucovorin. The reaction is strongly stimulated by copper ions at 1×10^{-4} *M*.

The enzyme is completely inhibited by cyanide or versene.

Assay. The action of inosinic transformylase can be followed by the determination of the quantities of the arylamine formed by means of the diazo reaction of Bratton and Marshall.[236n]

2. URICASE

History. The earliest observations on the power of mammalian tissues to destroy uric acid date back to Ascoli[237] and Wiener.[238] Schittenhelm[239] came

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[236i] W. Shive, W. W. Ackermann, M. Gordon, M. E. Getzendaner, and R. E. Eakin, *J. Am. Chem. Soc.* **69,** 725 (1947).

[236j] J. M. Buchanan and M. P. Schulman, *J. Biol. Chem.* **202,** 241 (1953).

[236k] J. G. Flaks and J. M. Buchanan, *J. Am. Chem. Soc.* **76,** 2275 (1954).

[236l] G. R. Greenberg, *Federation Proc.* **13,** 221 (1954).

[236m] G. R. Greenberg, *Federation Proc.* **13,** (1954), in press.

[236n] A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.* **128,** 537 (1939).

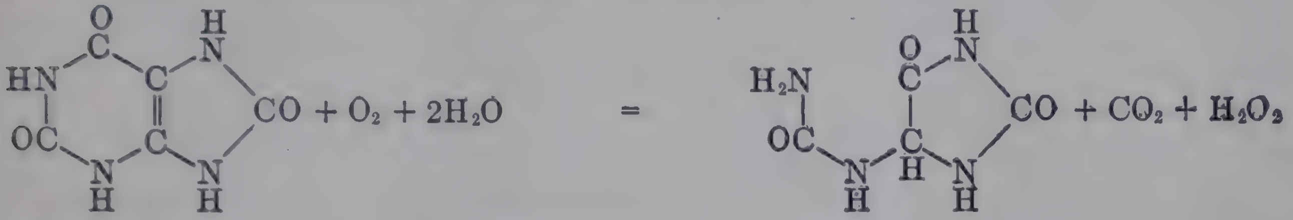
[237] G. Ascoli, *Pflügers Arch. ges. Physiol.* **72,** 340 (1898).

[238] W. Wiener, *Z. physiol. Chem.* **43,** 532 (1904).

[239] J. A. Schittenhelm, *Z. physiol. Chem.* **45,** 121 (1905).

to the conclusion that the oxidation of xanthine and that of uric acid were catalyzed by different enzymes and introduced the name uricase for the enzyme system responsible for the latter process. In 1907, Wiechowski[240] identified allantoin as the main end-product of enzymic uricolysis. This substance had been known much earlier as the main reaction product of oxidation of uric acid with permanganate. The first thorough study of the mechanism of uricase action was carried out by Batelli and Stern.[241]

Definition. Uricase is a uric acid oxidase which—under conditions resembling those in living cells—transforms uric acid in yields of more than 95% to racemic[242] allantoin. Under these conditions, the overall reaction closely approaches the equation given by Keilin and Hartree.[243-245]



Present Concepts Regarding the Mechanism of Enzymic Uricolysis. A comparison of the structures of uric acid and of allantoin shows that enzymic uricolysis is a complex process involving oxidation and a decarboxylation involving the opening of the pyrimidine ring of uric acid. This suggests the assumption that the transformation of uric acid to allantoin is a sequence of at least two reactions, one oxidative and one decarboxylating step. Since it is most unlikely that these two reactions are catalyzed by the same enzyme, one must either assume, according to Felix *et al.*[246] that uricase consists of a mixture of at least two enzymes, or that uric acid is oxidized enzymically to a labile intermediary product which subsequently yields allantoin by a nonenzymic degradation. At present, the latter alternative is favored as a working hypothesis. In the following, some current concepts regarding the nature of the labile intermediary compound will be briefly discussed.

Mechanism of the oxidation reaction. Bentley and Neuberger[247] demonstrated in experiments with O^{18} and H_2O^{18} that the oxygen of the hydrogen peroxide formed during uricolysis originated exclusively from gaseous oxygen. The oxidation of uric acid consists therefore in the transfer of two electrons from each uric acid molecule to molecular oxygen.

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[240] W. Wiechowski, *Beitr. chem. Path. Physiol.* **9,** 295 (1907).

[241] F. Batelli and L. Stern, *Biochem. Z.* **19,** 219 (1909).

[242] L. B. Mendel and H. D. Dakin, *J. Biol. Chem.* **7,** 153 (1916).

[243] D. Keilin and E. F. Hartree, *Proc. Roy. Soc. (London)* **B119,** 114 (1936).

[244] C. G. Holmberg, *Biochem. J.* **33,** 1901 (1939).

[245] J. N. Davidson, *Biochem. J.* **36,** 252 (1942).

[246] K. Felix, F. Scheel, and W. Schuler, *Z. physiol. Chem.* **215,** 258 (1929).

[247] R. Bentley and A. Neuberger, *Biochem. J.* **52,** 694 (1952).

According to Keilin and Hartree the quotient $CO_{2\text{formed}}:O_{2\text{consumed}}$ has the value of 1 with purified uricase preparations which are free of catalase. Crude uricase preparations are always contaminated with catalase, and their action results in an increased respiratory quotient.

Mechanism of the decarboxylation reaction. Progress in the understanding of uricase action was initiated by three approaches: (1) The direct experimental proof for the origin of the carbon dioxide from the 6-C atom of uric acid. This evidence was furnished by Bentley and Neuberger[247] by the enzymic uricolysis of 6-C^{14}-labelled uric acid. (2) The experimental separation of uricase action into its oxidation and decarboxylation phases. The feasibility of such a separation was first demonstrated in 1929 by Felix, Scheel, and Schuler,[246] who found that, at pH 8.9, the oxygen consumption was much more rapid than the carbon dioxide formation. A further important advance was Klemperer's observation[248] (1945) that borate ions have a specific inhibitory effect on the decarboxylation phase and allantoin formation of uricase action. The study of enzymic uricolysis in borate buffers has become an indispensable method for the exploration of the mechanism of uricase action. (3) The oxidation of uric acid with alkaline permanganate which also results in the formation of allantoin is a useful model reaction in the search for the unstable intermediary of the biological formation of allantoin.[249-251]

Nature of the unstable intermediary product of the enzymic oxidation of uric acid (see also Chapter 26). Cavalieri and Brown[252] found that 1,3-N^{15}-labelled uric acid yielded an allantoin which was equally labelled in both ureido groups. The same holds true for allantoin isolated from the urine of rats fed with 1,3-N^{15}-labelled uric acid. On the basis of these observations Brown *et al.*[253] suggested an oxidation product of symmetrical structure as the immediate precursor of allantoin. A similar postulate for the permanganate oxidation of uric acid had already been made by Behrend,[249] who explained by this assumption earlier observations by Fischer and Ach.[254] These authors found that permanganate oxidation of 1-methyluric acid and 7-methyluric acid yielded 3-methylallantoin, whereas the oxidation of both 3-methyl uric acid and 9-methyluric acid yielded 1-methylallantoin.

The postulated symmetrical intermediary (Fig. 9) formed during permanganate oxidation was isolated by Schuler and Reindel[251] as the trisilver

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[248] F. W. Klemperer, *J. Biol. Chem.* **160,** 111 (1945).

[249] R. Behrend, *Ann.* **333,** 141 (1904).

[250] R. Behrend and R. Schultz, *Ann.* **365,** 21 (1909).

[251] W. Schuler and W. Reindel, *Z. physiol. Chem.* **208,** 248 (1932).

[252] L. F. Cavalieri and G. B. Brown, *J. Am. Chem. Soc.* **70,** 1242 (1948).

[253] G. B. Brown, P. M. Roll, A. A. Plentl, and L. F. Cavalieri, *J. Biol. Chem.* **172,** 469 (1948).

[254] E. Fischer and F. Ach, *Ber.* **32,** 2745 (1899).

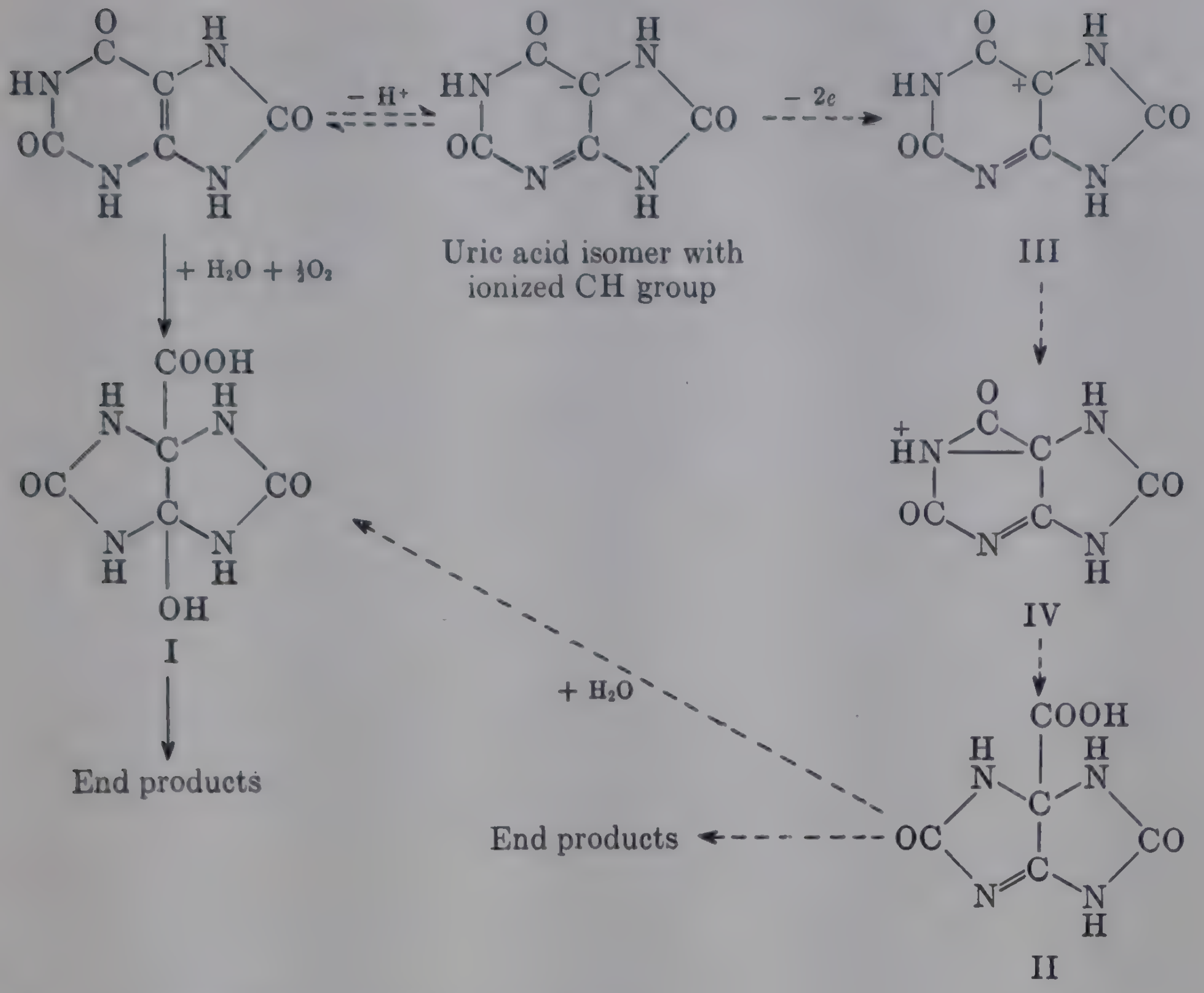

FIG. 9. Intermediary steps in uricolysis.
———— Schuler and Reindel's concept
---------- Bentley and Neuberger's concept

salt of hydroxyacetylenediureidocarboxylic acid (I). On heating on a boiling water bath for 30 minutes, in 50% acetic acid, the silver salt yields allantoin in 50% yield; by heating in alkali under similar conditions, the symmetric ring compound I is oxidized to uroxanic acid (diureidomalonic acid, II) with opening of both rings.

Schuler and Reindel reported[255] the isolation of a silver salt identical with that of substance I from digests obtained by enzymic uricolysis. The initial increase of the acidity of such digests also favors the assumption of the intermediary formation of a carboxyl compound prior to the formation of carbon dioxide and allantoin.

Bentley and Neuberger raised two objections to the assumption that hydroxyacetylenediureidocarboxylic acid (I) would be the intermediary product of enzymic uricolysis: (1) the transient increases of the light absorptions at 260 and 325 mμ during the enzymic uricolysis in phosphate buffers (Praetorius[256]); (2) the relatively drastic conditions required for the nonenzymic decarboxylation of I to allantoin.

[255] W. Schuler and W. Reindel, *Z. physiol. Chem.* **215**, 258 (1933).
[256] E. Praetorius, *Biochim. et Biophys. Acta* **2**, 602 (1949).

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[248] F. W. Klemperer, *J. Biol. Chem.* **160,** 111 (1945).

[249] R. Behrend, *Ann.* **333,** 141 (1904).

[250] R. Behrend and R. Schultz, *Ann.* **365,** 21 (1909).

[251] W. Schuler and W. Reindel, *Z. physiol. Chem.* **208,** 248 (1932).

[252] L. F. Cavalieri and G. B. Brown, *J. Am. Chem. Soc.* **70,** 1242 (1948).

[253] G. B. Brown, P. M. Roll, A. A. Plentl, and L. F. Cavalieri, *J. Biol. Chem.* **172,** 469 (1948).

[254] E. Fischer and F. Ach, *Ber.* **32,** 2745 (1899).

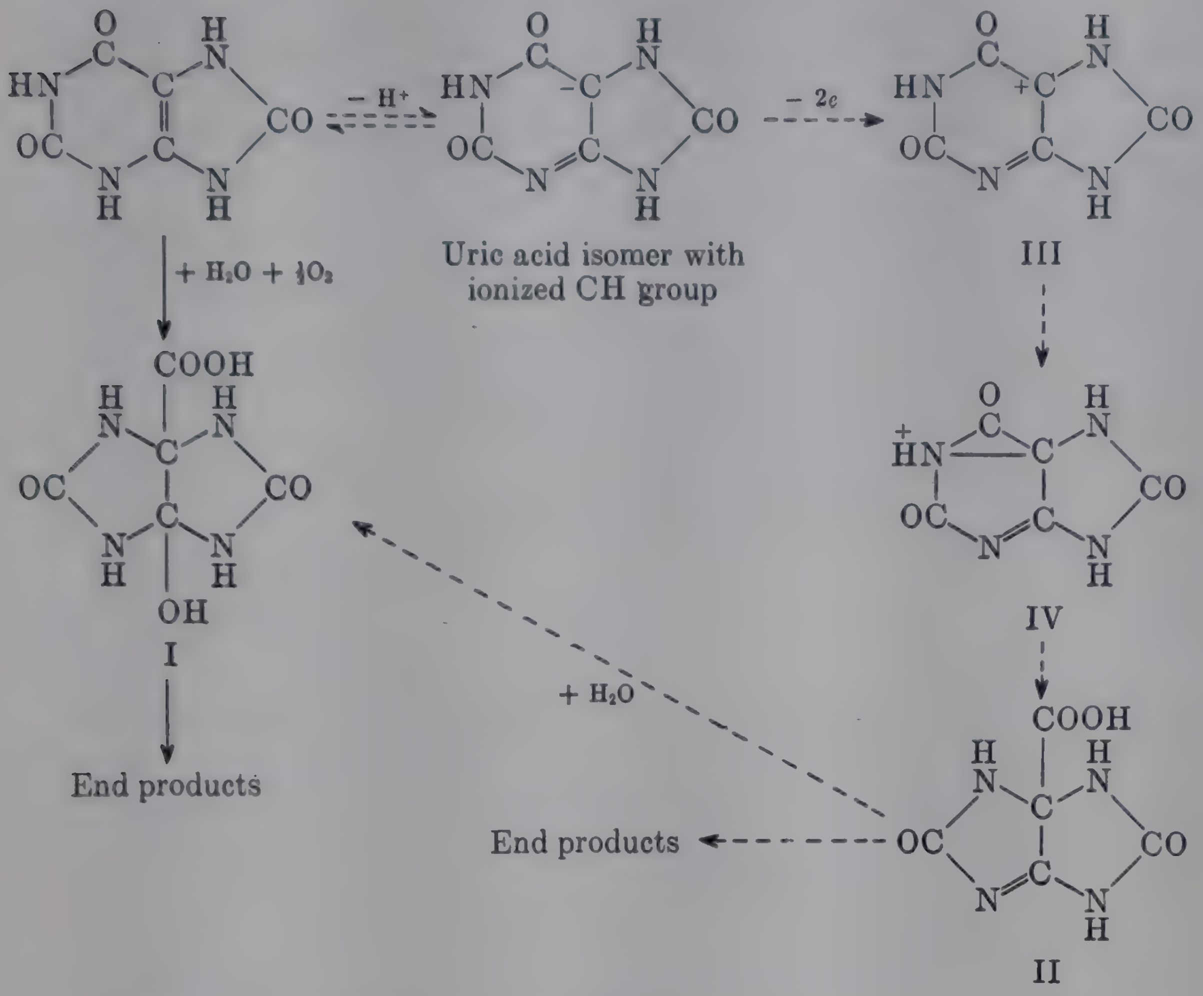

FIG. 9. Intermediary steps in uricolysis.
——— Schuler and Reindel's concept
--------- Bentley and Neuberger's concept

salt of hydroxyacetylenediureidocarboxylic acid (I). On heating on a boiling water bath for 30 minutes, in 50% acetic acid, the silver salt yields allantoin in 50% yield; by heating in alkali under similar conditions, the symmetric ring compound I is oxidized to uroxanic acid (diureidomalonic acid, II) with opening of both rings.

Schuler and Reindel reported[255] the isolation of a silver salt identical with that of substance I from digests obtained by enzymic uricolysis. The initial increase of the acidity of such digests also favors the assumption of the intermediary formation of a carboxyl compound prior to the formation of carbon dioxide and allantoin.

Bentley and Neuberger raised two objections to the assumption that hydroxyacetylenediureidocarboxylic acid (I) would be the intermediary product of enzymic uricolysis: (1) the transient increases of the light absorptions at 260 and 325 mμ during the enzymic uricolysis in phosphate buffers (Praetorius[256]); (2) the relatively drastic conditions required for the nonenzymic decarboxylation of I to allantoin.

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[255] W. Schuler and W. Reindel, *Z. physiol. Chem.* **215**, 258 (1933).

[256] E. Praetorius, *Biochim. et Biophys. Acta* **2**, 602 (1949).

These authors proposed instead for the intermediary a hypothetical structure which represents a dehydration product of Schuler and Reindel's carboxylic acid, and which contains a tautomeric double bond in one of th cyclic ureide moieties (II). According to these authors, uric acid is dehydrogenated to a carbonium compound (III) which isomerizes to an unstabl cyclic quaternary ammonium compound (IV). This is spontaneousl hydrolyzed to II.

Evidence in favor of the nonenzymic nature of the decarboxylation reactio of uricolysis. The recent hypotheses regarding the nature of the intermediary substance or substances[256] of enzymic uricolysis depend largely on the assumption of a nonenzymic terminal step of decarboxylation. Three reasons are usually given in favor of the nonenzymic nature of this step and of the enzymic homogeneity of uricase: (1) the preservation of the complete enzyme system through numerous fractionation steps in all purificatio procedures; (2) the chemical lability of the intermediary products; (3) the observations initiated by Klemperer,[248] who found that, in addition to allantoin, other end products are obtained during the enzymic uricolysis in borate buffer. In such digests, urea, allantoin, alloxanic acid, and oxaluric acid were recently identified by chromatographic procedures.[257]

It is clear that none of these three reasons excludes the possibility of the presence of a specific decarboxylase in the uricase preparations, although the heterogeneity of the end products obtained in borate buffers renders their direct enzymic formation unlikely. The spontaneous degradation of the labile intermediary in the presence of borate could be explained by the presence of a borate-sensitive decarboxylase in the uricase system.

Specificity. Uricase is a highly specific enzyme, uric acid being the only purine derivative which is oxidized under its influence. Some uric acid derivatives, however, are competitive inhibitors of uricase, such as 7-methyluric acid and 1,3,7-trimethyluric acid.[243] Owing to this high specificity, uricase is used as a specific reagent for the determination of uric acid. Many of the recent procedures for the purification[258-266] of uricase were devised for this purpose.

pH Optimum. Keilin and Hartree[243] found the pH optimum for the action

[257] E. L. Canellakis and P. P. Cohen, *Federation Proc.* **13,** 189 (1954).

[258] C. G. Holmberg, *Biochem. J.* **33,** 1901 (1939).

[259] K. I. Altman, K. Sunell, and G. E. L. Barron, *Arch. Biochem.* **21,** 158 (1949).

[260] M. B. Blauch and F. C. Koch, *J. Biol. Chem.* **130,** 443 (1939).

[261] H. A. Bulger and H. E. Johns, *J. Biol. Chem.* **140,** 427 (1941).

[262] N. K. Schaffer, *J. Biol. Chem.* **153,** 163 (1944).

[263] W. D. Block and N. C. Geib, *J. Biol. Chem.* **168,** 747 (1947).

[264] H. M. Kalckar, *J. Biol. Chem.* **167,** 429 (1947).

[265] H. Silverman and I. Gubernick, *J. Biol. Chem.* **167,** 363 (1947).

[266] E. Leone, *Biochem. J.* **54,** 393 (1953).

of purified uricase in the region of 9.2. Since uricase action is usually assayed by determination of the oxygen uptake or by that of uric acid disappearance, these observations refer to the oxidative phase of uricase action. Felix *et al.*,[246] who worked with much cruder enzyme preparations, found the optimum pH of the oxidative phase at pH 9.2 and that of the decarboxylation phase at pH 9.9. Owing to the still problematic nature of the "decarboxylation reaction," the question of the pH optimum of the decarboxylation requires further clarification.

Inhibitors. Uricase action is inhibited to an extent of 94% in presence of 1×10^{-3} *M* potassium cyanide, and to an extent of 83% in the presence of 3×10^{-5} *M* potassium cyanide.[243,267,268] The cyanide inhibition is completely reversed by dialysis.[245]

Carbon monoxide is without influence on uricase according to Davidson.[245]

Many heavy metals (Cu, Fe, Mn, Zn, Co, Ni) have considerable inhibitory effects at concentrations in the range of 0.001 *M*.[245]

Pyrophosphates, fluorides, and urethan have no inhibitory effect. In the presence of sodium azide the activity decreases by 28% at pH 8 and by 40% at pH 6.8.

Role of Heavy Metals. The possible significance of the zinc (0.1%) and iron (0.2%) contents of purified uricase preparations is not yet clear.[245] Praetorius concluded from the failure of British Anti-Lewisite (BAL) to interfere with uricase action that the zinc content of uricase preparations has no connection with their activity.[269]

IX. Enzymes Involving the Opening of the Pyrimidine Ring

Intact animals metabolize administered uracil and thymine with the formation of urea[270-274] or of β-amino acids[274a] (see also Chapter 26). The catabolism of pyrimidines in cell-free systems has so far been studied mainly on bacterial enzymes. No observations on cell-free systems from animal sources are as yet available, but the results obtained with tissue slices offer interesting correlations with the mechanisms demonstrated for the action of bacterial enzymes.

[267] H. R. Fosse, *Compt. rend.* **191,** 1153 (1930).
[268] R. Truszkowski, *Biochem. J.* **24,** 1340 (1930).
[269] E. Praetorius, *Biochim. et Biophys. Acta* **2,** 590 (1948).
[270] H. Steudel, *Z. physiol. Chem.* **32,** 285 (1901).
[271] L. R. Cerecedo, *J. Biol. Chem.* **93,** 269 (1931).
[272] L. B. Mendel and V. C. Myers, *Am. J. Physiol.* **26,** 77 (1910).
[273] D. W. Wilson, *J. Biol. Chem.* **56,** 215 (1923).
[274] H. Deuel, *J. Biol. Chem.* **60,** 749 (1924).
[274a] K. Fink, R. B. Henderson, and R. M. Fink, *J. Biol. Chem.* **197,** 441 (1952).

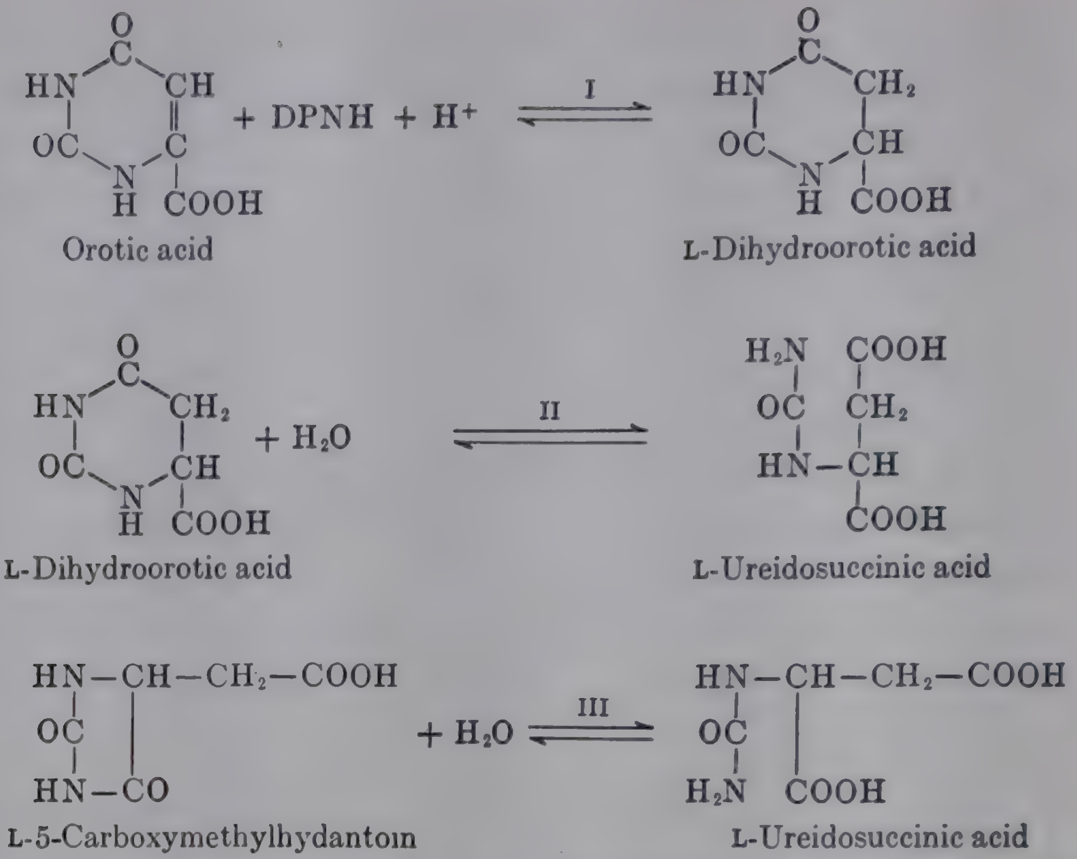

FIG. 10. Action of dihydroorotic acid dehydrogenase (I), dihydroorotase (II), and 5-carboxymethylhydantoinase (III).

1. REVERSIBLE DEGRADATION OF OROTIC ACID BY BACTERIAL ENZYMES

The enzymes involved in metabolism of orotic acid are of great interest since this naturally occurring pyrimidine derivative is utilized for the biosynthesis of nucleic acid pyrimidine groups of various organisms (see Chapter 23).

Lieberman and Kornberg[274b,c] found in the soil organism *Zymobacterium oroticum*[274d] an enzyme system capable of catalyzing the reversible transformation of orotic acid to L-ureidosuccinic acid (Fig. 10). The first step of this overall reaction is the hydrogenation of orotic acid to dihydroorotic acid under the influence of a specific enzyme, dihydroorotic acid dehydrogenase.

Observations with isotopes leave no doubt that formation of orotic acid in the mammalian organism follows the same pathway. The enzymic synthesis of ureidosuccinic acid from aspartic acid, carbon dioxide and ammonia was recently demonstrated by Reichard[274e] with cell-free rat liver preparations. The reaction requires adenosine triphosphate and magnesium ions.

a. Dihydroorotic Acid Dehydrogenase[274b]

Dihydroorotic acid dehydrogenase was separated from cell-free extracts of *Zymobacterium oroticum*[274d] by precipitation with protamine and fractionation of the solution of the protamine precipitate with ammonium sulfate.

[274b] I. Lieberman and A. Kornberg, *Biochim. et Biophys. Acta* **12,** 223 (1953).

[274c] I. Lieberman and A. Kornberg, *J. Biol. Chem.* **207,** 911 (1954).

[274d] J. T Wachsman and H. A. Barker, *J. Bacteriol.* **68,** 400 (1954).

[274e] P. Reichard, *Acta Chem. Scand.* **8,** 795 (1954).

The enzyme reaction requires either the presence of stoichiometric amounts of reduced DPN or of catalytic amounts of DPN in the presence of a hydrogen-donor system.

Specificity. Dihydroorotic acid dehydrogenase is strictly specific for orotic acid. Uracil, cytosine, thymine, and 5-methylcytosine are inert and have no influence on the rate of hydrogenation of orotic acid.

pH Optimum. The optimal pH range is between pH 6.4 and 6.5.

The *Michaelis-Menten constant* has the value of 1.1×10^{-4} M concentration of orotic acid.

The presence of the enzyme in other microorganisms is suggested by the fact that dihydroorotic acid is a growth factor for *Lactobacillus bulgaricus.*

b. Dihydroorotase[274c]

The reversible hydrolysis of dihydroorotic acid to ureidosuccinic acid is catalyzed by dihydroorotase which is present in cell-free extracts of *Zymobacterium oroticum.*

Equilibrium. Lieberman and Kornberg measured the equilibrium of the reaction by using C^{14}-labelled substrates and by determining the total radioactivity of the components of the incubation mixture after chromatographic separation. At pH 6.1, the ratio of ureidosuccinate to dihydroorotate at equilibrium is approximately 1.9.

c. 5-Carboxymethylhydantoinase[274c]

It is interesting that the formation of dihydroorotic acid is not the only type of enzymic ring-closure which ureidosuccinic acid undergoes in cell-free extracts of *Zymobacterium oroticum.* These extracts contain a specific enzyme, 5-carboxymethylhydantoinase, which catalyzes the reversible ring-closure between the ureido group and the vicinal carboxy group to L-5-carboxymethylhydantoin.

Equilibrium. The equilibrium constant is similar to that of dihydroorotase, amounting to the value of 1.9 for the proportion between 5-carboxymethylhydantoin and ureidosuccinate.

The specificity of 5-carboxymethylhydantoinase has not yet been investigated. Liver homogenates which hydrolyze hydantoin[274f] were found to be without activity toward L-5-carboxymethylhydantoin.

2. CONVERSION OF DIHYDROURACIL AND DIHYDROTHYMINE TO β-AMINO ACIDS BY TISSUE SLICES

The reductive pathway of the reversible degradation of orotic acid under the influence of the enzyme system of *Zymobacterium oroticum* has an interesting parallel in the behavior of liver and kidney slices toward dihydrothymine and dihydrouracil. Fink *et al.*[274g] demonstrated by paper

[274f] F. Bernheim and M. Bernheim, *J. Biol. Chem.* **163,** 683 (1946).

[274g] R. M. Fink, K. Fink, and R. B. Henderson, *J. Biol. Chem.* **201,** 349 (1953).

chromatography the conversion of dihydrouracil to β-alanine and that o dihydrothymine to β-aminoisobutyric acid by slices of liver and—to lesser extent—of kidney. Slices obtained from rat, rabbit, guinea pig, cow and monkey gave similar results.

Specificity. Only slight formation of β-aminoisobutyric acid occurred whe thymine was used as substrate. No production of β-amino acids was detected with uracil, uridine, uridylic acid, cytosine, 5-methylcytosine, and orotic acid.

The excretion of β-aminoisobutyric acid by rats after administration o thymine, of deoxyribonucleic acid, or—to a greater extent—of dihydrothymidine suggests the assumption that the dihydropyrimidines are physiological intermediaries in this pathway of mammalian pyrimidine catabolism Furthermore, dihydrouracil has been isolated from beef spleen by Funk *e al.*[274h]

3. Degradation of Uracil and Thymine by Bacterial Enzymes

a. Bacterial Oxidase of Uracil and Thymine (Fig. 11)

Hayaishi and Kornberg,[275] as well as Wang and Lampen,[276] demonstrated in cell-free extracts of three different species of soil bacteria the presence of an enzyme which catalyzed the oxidation of uracil and thymine. The oxidation product of uracil is barbituric acid, that of thymine, 5-methylbarbituric acid. Since the determinations of the Michaelis constants gave practically identical results for the affinities to uracil, whether determined with uracil as substrate, or with uracil as competitive inhibitor of thymine oxidation, it appears that both pyrimidines are oxidized by the same enzyme.

Specificity. The oxidase is very specific for uracil, thymine, and 5-amino uracil. Barbituric acid, isobarbituric acid, 5-methylbarbituric acid, 6-methyluracil, dihydrothymine, dihydrouracil, 2-thiouracil, 2-thiothymine, cytosine, and methylcytosine are inert.

pH Optimum. The optimal pH is approximately at 8.5; at neutral reaction only one-tenth of the optimal activity was observed.

Mechanism. The presence of methylene blue is required for the action of the enzyme; so far it could not be replaced by the physiological hydrogen acceptors or mediators of electron transfer.

Michaelis Constants. The enzyme–substrate dissociation constants are $0.35 \times 10^{-4}\ M$ for thymine and $1.3 \times 10^{-4}\ M$ for uracil.

b. Barbiturase (Fig. 12)

The end-products of the oxidation of uracil by the intact bacteria mentioned in the previous section are carbon dioxide, ammonia, and water.

[274h] C. Funk, A. J. Merritt, and A. Ehrlich, *Arch. Biochem. and Biophys.* **35,** 468 (1952).

[275] O. Hayaishi and A. Kornberg, *J. Biol. Chem.* **197,** 717 (1952).

[276] T. P. Wang and J. O. Lampen, *J. Biol. Chem.* **194,** 785 (1952).

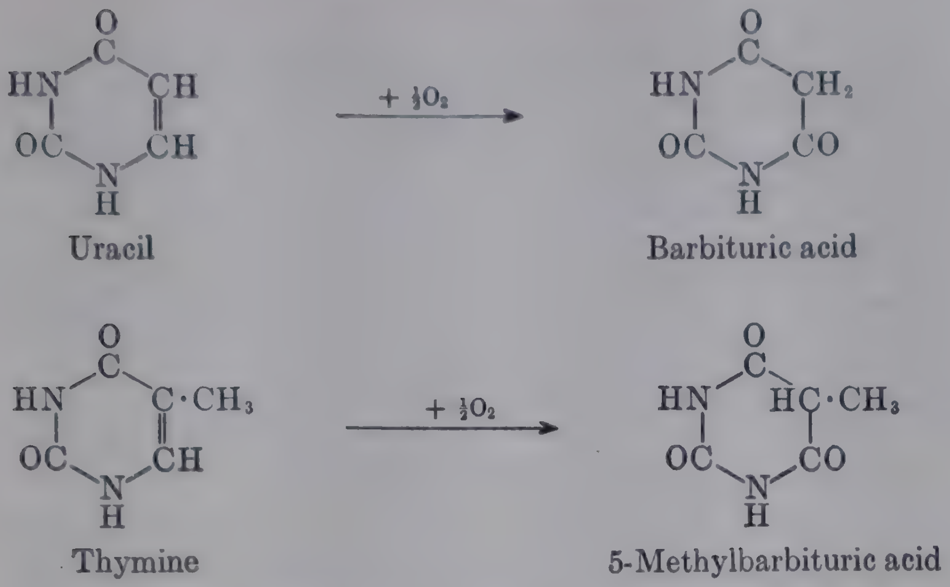

FIG. 11. Bacterial oxidation of uracil and thymine.

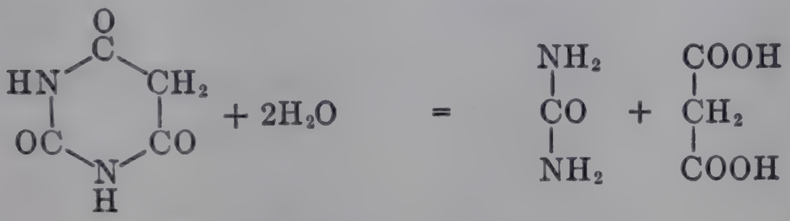

FIG. 12. Action of barbiturase.

Hayaishi and Kornberg succeeded in obtaining from cell-free extracts of mycobacteria (obtained at pH 6.65) besides urease an enzyme which catalyzes the cleavage of barbituric acid into urea and malonate. The enzyme could be separated from most of the urease by purification with protamine sulfate. In crude extracts, the urease activity could be selectively suppressed by the addition of silver ions in 0.5×10^{-4} M concentration.

Specificity. Barbiturase is a highly specific enzyme. 5-Methylbarbituric acid, orotic acid, barbital, pentobarbital, 2-thiobarbituric acid, and isobarbituric acid are inert.

pH Optimum. The optimal range of the activity is between pH 8 and pH 9.

Michaelis Constant. The approximate K_M value is 3.4×10^{-4} M.

The enzymic mechanism of the final degradation of 5′-methylbarbiturate which is rapidly metabolized by the intact bacteria under aerobic conditions is as yet unknown.

X. Some Data Concerning the Intracellular Distribution of Enzymes of Nucleic Acid Metabolism

Owing to the significance of nucleic acids as constituents of vital structural elements of cells (chromosomes, nucleoli, mitochondria, Nissl bodies), the intracellular distribution of the enzymes of nucleic acid metabolism is of great interest and is discussed also in Chapters 18 and 21. The interpretation of the available data, however, requires caution because variou interfering factors must be considered. Some of those factors are: (1) Th presence of abundant amounts of substrates in some cell fractions, particularly in studies concerning deoxyribonuclease and ribonuclease. (2) The possibility of absorption of enzymes by particles during homogenization. Thi source of error is serious when citric acid procedures are employed for th isolation of nuclei. (3) The influence of the ionic environment and other activators and inhibitors on the action of the enzymes.

1. Deoxyribonuclease[87a,119,123,277-279]

According to Allfrey and Mirsky,[87a] deoxyribonuclease I appears to be a specific digestive enzyme of pancreas. It is not found in significant concentrations in other tissues, but its activity in pancreas (as differentiated by means of its pH optimum) exceeds that of other deoxyribonucleases more than 200-fold. It was found in high concentration in the pancreas fistula juice of a dog, whereas no appreciable amounts of other deoxyribonucleases could be demonstrated.

Deoxyribonucleases of acid pH optima were found in many tissues in which they are mainly associated with the cytoplasmic fraction, particularly with the mitochondria (Chapter 18). In contrast to some earlier reports, it appears now that the nuclei of liver, heart, and spleen have little if any deoxyribonuclease activity (see also Chapter 21).

Kowlessar *et al.*[280,281] found that considerable amounts of deoxyribonucleases of acid as well as alkaline pH optima are excreted in the urine by mice which had been irradiated with lethal doses of X-rays. This suggests the liberation of intracellular nucleases under conditions producing extensive cell damage.

2. Ribonuclease

According to Roth,[282] ribonucleases of acid as well as alkaline pH optima occur in many tissues although the concentration of ribonuclease I in pancreas exceeds by far the ribonuclease activity of any other animal tissue. In liver, the mitochondrial fraction contains a larger part of "acid" and "alkaline" ribonuclease activity than do other fractions of the homogenates. Liver nuclei contain small, but measurable, amounts of ribonuclease activity

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[277] W. C. Schneider and G. H. Hogeboom, *J. Biol. Chem.* **198,** 155 (1952).

[278] R. J. Neff, Thesis, Univ. of Missouri, 1951.

[279] K. Land, G. Siebert, I. Baldus, and A. Corbet, *Experientia* **6,** 59 (1950).

[280] O. D. Kowlessar, K. I. Altman, and I. H. Hempelman, *Arch. Biochem. and Biophys.* **43,** 233 (1953).

[281] O. D. Kowlessar, K. I. Altman, and I. H. Hempelman, *Nature* **172,** 867 (1953).

[282] J. S. Roth, *J. Biol. Chem.* **208,** 181 (1954).

with a single optimum at pH 7.8. Miller and Kozloff[283] demonstrated the presence of ribonuclease activity in the nuclear fraction of chicken erythrocytes obtained by saponin hemolysis. These authors believe that the ribonuclease activity of the nuclei is localized in the nuclear membrane since it is not increased by freezing and thawing of the enzyme preparation.

3. Intracellular Localization of Deoxyribonuclease I and Ribonuclease I in Pancreas

The crystallized ribo- and deoxyribonucleases of the pancreas offer the experimental advantage that specific antibiodies for these two enzymes can be prepared. Such antibodies were used by Marshall for studies on the intracellular distribution of ribonuclease I and deoxyribonuclease I by histochemical application of the specific antibodies labelled with fluorescein.[284,285] Ribonuclease I and deoxyribonuclease I were found in the zymogen granules and in the cytoplasm, but not in mitochondria and nuclei. Schneider and Hogeboom[277] found ribo- and deoxyribonuclease activity in liver mitochondria. Probably the mitochondrial nucleases are not identical with the crystallized enzymes of pancreas.

4. Other Enzymes of Nucleic Acid Metabolism

According to Stern *et al.*,[286] adenosine deaminase, guanase, and nucleoside phosphorylase are present in high concentrations in nuclei of many tissues, whereas nucleotide phosphatases and uricase are present only in relatively small amounts if at all. This question is discussed further in Chapters 18 and 21.

XI. Addendum

(*Concerning the Enzymic Formation of Nucleoside Di- and Triphosphates*)

When the organization of Chapter 15 was undertaken it was decided to exclude enzyme reactions involving the pyrophosphoryl groups of nucleoside polyphosphates since their discussion seemed to be pertinent to the field of energy metabolism rather than to that of nucleic acids. During the last year, however, the presence of polyphosphates of various ribonucleosides in animal tissues was demonstrated by Schmitz, Hurlbert and Potter.[287] All these compounds are phosphorylated in their 5′-positions. Conceivably, these substances might be intermediaries in the biosynthesis of nucleic acids although no direct evidence in favor of such a hypothesis exist as yet.

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[283] Z. B. Miller and L. M. Kozloff, *J. Biol. Chem.* **170,** 105, (1947).

[284] J. M. Marshall, *Exptl. Cell Research* **6,** 240 (1954).

[285] A. H. Coons, H. J. Creech, R. N. Jones, and E. Berliner, *J. Immunol.* **45,** 159 (1942).

[286] H. Stern, V. G. Allfrey, A. E. Mirsky, and H. Laetren, *J. Gen. Physiol.* **35,** 559 (1952).

[287] H. Schmitz, R. B. Hurlbert, and V. R. Potter, *J. Biol. Chem.* **209,** 41 (1954).

Several enzymes catalyzing the formation of nucleoside polyphosphate are known. The pathways involved include transphosphorylation betwee nucleoside polyphosphates as well as reactions in which inorganic phosphat participates in the enzymatic synthesis.

(1) *Diphosphokinase reactions:* Berg and Joklik found that enzyme fractions of muscle and of bakers' and brewers' yeast mediate the reversibl transphosphorylation between adenosine triphosphate and hypoxanthosin diphosphate with formation of adenosine diphosphate and hypoxanthosin triphosphate.[288]

(2) *Reaction of the myokinase*[289] *type* (Transphosphorylations betwee nucleoside triphosphates and nucleoside-5′-monophosphates). According t Lieberman, Kornberg, and Simms,[290] cell free yeast preparations catalyz a transphosphorylation between adenosine triphosphate or uridine triphosphate, on the one hand, and 5′-uridylate on the other hand, to the diphosphates (uridine diphosphate is inert in this system).

(3) *Formation of guanosine triphosphate or hypoxanthosine triphosphat with participation of inorganic phosphate.* Sanadi *et al.*[291] found that the enzymic synthesis of adenosine triphosphate from adenosine diphosphate, inorganic phosphate and succinyl coenzyme A requires the intermediary formation of either guanosine- or inosine triphosphate from the respective diphosphates. Guanosine-, as well as hypoxanthosine triphosphate, in turn can act as phosphate donors in reversible enzymic phosphorylations of other mono- or dinucleotides.

In some cases[288-290] it has been demonstrated that the enzymes catalyzing transphosphorylations between nucleoside triphosphates and nucleoside diphosphates are different from those catalyzing transphosphorylations between nucleoside tri- and nucleoside monophosphates. The discussion of such reactions in the three groups of this chapter, however, does not imply a general validity of this classification with regard to enzyme specificity.

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[288] P. Berg and W. K. Joklik, *Nature* **172,** 1008 (1953).

[289] S. P. Colowick and H. M. Kalckar, *J. Biol. Chem.* **148,** 117 (1943).

[290] I. Lieberman, A. Kornberg, and E. S. Simms, *J. Am. Chem. Soc.* **76,** 3608 (1954).

[291] R. Sanadi, D. M. Gibson, and P. Ayengar, *Biochim. et Biophys. Acta* **14,** 434 (1954).

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Figures in parentheses are reference numbers. They are included to assist the reader in locating a reference when a work is cited, but the author's name is not mentioned.

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